REMARKS

By the present amendment, claims 1-31 have been cancelled, without prejudice or disclaimer to the subject matter disclosed therein, and claims 32-37 have been added. Claims 32-37 were pending in parent Application No. 10/232,858 filed September 3, 2002 as claims 38-43 (previously numbered claims 16-21), and have been cancelled from that application and re-presented in the present application in order to expedite the declaration of the Interference previously requested between the subject matter of these claims and U.S. Patent No. 6,284,740 ('740). The request for interference was made in the Preliminary Amendment filed September 3, 2002 in parent Application No. 10/232,858, and is herein reiterated.

Also submitted herewith is a substitute specification, a substitute sequence listing, and formal drawings. No new matter is added by any of these submissions.

Support for current claims 32-37 may be found in the original specification (grand-parent Application No. 08/915,004, filed August 20, 1997, at, for example, page 4, lines 1-4; page 5, line 12, to page 6, line 16; page 7, lines 5-8; page 11, lines 13-24; and SEQ ID NOs: 5 and 6. Furthermore, with reference to 37 C.F.R. § 1.607(a)(5), Applicants respectfully direct the Examiner's attention to the support in the original specification as well as the substitute specification for claims 32-34:

Claim	**Support in specification as originally filed**	**Support in substitute specification**
32. A method of improving decreased bone mass in a human comprising:	Page 11, lines 13-14: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass..." Page 11, lines 19-21: "The preparation . . . is safely administered to human..."	Page 10, lines 18-19: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass..." Page 10, lines 23-24: "The preparation . . . is safely administered to humans..." [1]

[1] *See also* Applicants' Japanese Patent Application No. 054977 (1995), filed February 20, 1995 ("Priority Application No. 054977"), an English translation of which was attached to the Preliminary Amendment in parent Application No. 10/232,858 as **Annex B**, and from which priority is claimed. At page 10, lines 2-10, the translation states that "[t]he OCIF protein of the invention is useful as a pharmaceutical ingredient[]for treating or improving decreased bone mass such as osteoporosis, decreases of bone mass such as an abnormal bone metabolism. . . . The

providing a pharmaceutical preparation containing as an active ingredient OCIF protein encoded by SEQ ID NO:6 and	Page 11, lines 19-21: "The preparation contains the OCIF protein of the present invention as an effective ingredient...." Page 28, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in sequence number 6...."	Page 10, lines 23-24: "The preparation contains the OCIF protein of the present invention as an effective ingredient...." [2] Page 21, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in SEQ ID NO: 6...."[3]
administering the preparation containing OCIF protein so as to effect the improvement in decreased bone mass.	Page 11, lines 13-14: "The OCIF protein of the invention is useful as a pharmaceutical ingredients for treating or improving decreased bone mass..." Page 11, lines 19-21: "The preparation contains the OCIF protein of the present invention as an effective ingredient..."	Page 10, lines 18-19: "The OCIF protein of the invention is useful as a pharmaceutical ingredient for treating or improving decreased bone mass..."[4] Page 10, lines 23-24: "The preparation contains the OCIF protein of the present invention as an effective ingredient..." [5]
33. A method of using OCIF protein for improvement of decreased bone mass in a human comprising:	Page 11, lines 13-14: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass..." Page 11, lines 19-21: "The preparation . . . is safely administered to human...	Page 10, lines 18-19: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass. . . ." Page 10, lines 23-24: "The preparation . . . is safely administered to humans..." [6]
providing a pharmaceutical preparation for introducing	Page 11, lines 19-21: "The preparation contains the OCIF	Page 10, lines 23-24: "The preparation contains the OCIF

preparation contains the OCIF protein of the present invention as an efficacious ingredient and is safely administered to human." *See* additionally Applicants' Japanese Patent Application No. 207508 (1995), filed July 21, 1995 ("Priority Application No. 207508"), an English translation of which was attached to the Preliminary Amendment in parent Application No. 10/232,858 as **Annex C**, and from which priority is claimed, at page 12, line 18 through page 13, line 4.

[2] *See* Footnote 1, *supra.*

[3] *See* Priority Application No. 207508 at example 11, page 29, lines 18-19: "[t]he determined base sequence of OCIF is shown in the sequence No. 5 . . . ." [In the present Application, this same sequence is referred to as SEQ ID NO: 6].

[4] See Footnote 1, *supra.*

[5] See Footnote 1, *supra.*

[6] See Footnote 1, *supra.*

OCIF protein encoded by SEQ ID NO:6 into said human,	protein of the present invention as an effective ingredient. . ." Page 28, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in sequence number 6. . ."	protein of the present invention as an effective ingredient. . ."[7] Page 21, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in SEQ ID NO: 6. . ."[8]
and administering said preparation to said human so as to effect said improvement of said decreased bone mass.	Page 11, lines 13-14: "The OCIF protein of the invention is useful as a pharmaceutical ingredient for treating or improving decreased bone mass. . . ." Page 11, lines 19-21: "The preparation contains the OCIF protein of the present invention as an effective ingredient . . ."	Page 10, lines 18-19: "The OCIF protein of the invention is useful as a pharmaceutical ingredient for treating or improving decreased bone mass. . . ."[9] Page 10, lines 23-24: "The preparation contains the OCIF protein of the present invention as an effective ingredient . . ."[10]
34. A method of increasing levels of OCIF protein in a human comprising	Page 11, lines 13-14: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass. . ." Page 11, lines 19-21: "The preparation . . . is safely administered to humans . . ."	Page 10, lines 18-19: "The OCIF protein of the invention is . . . for treating or improving decreased bone mass. . ." Page 10, lines 23-24: "The preparation . . . is safely administered to humans . . ." [11]
administering to said human said OCIF protein encoded by SEQ ID NO:6,	Page 11, lines 19-21: "The preparation contains the OCIF protein of the present invention as an effective ingredient. . ." Page 28, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in sequence number 6. . ."	Page 10, lines 23-24: "The preparation contains the OCIF protein of the present invention as an effective ingredient. . ."[12] Page 21, Example 12: "The nucleotide sequence of the OCIF cDNA is shown in SEQ ID NO: 6. . ."[13]

[7] See Footnote 1, *supra*.

[8] See Footnote 3, *supra*.

[9] See Footnote 1, *supra*.

[10] See Footnote 1, *supra*.

[11] See Footnote 1, *supra*.

[12] See Footnote 1, *supra*.

[13] See Footnote 3, *supra*.

wherein such administration results in an increase in the level of OCIF protein and wherein the increase in OCIF protein results in an increased bone density.	Page 11, lines 13-14: "The OCIF protein of the invention is useful as a pharmaceutical ingredients for treating or improving decreased bone mass. . . ."	Page 10, lines 18-19: "The OCIF protein of the invention is useful as a pharmaceutical ingredient for treating or improving decreased bone mass. . . ."[14]

Submitted herewith is a substitute specification and abstract for the above-identified application, entry of which is respectfully requested. *See* MPEP § 608. The substitute specification has been amended to include cross reference to related applications and incorporation of sequence listing sections. The substitute specification also corrects alleged defects in parent application number 10/232,858, corrects minor typographical and grammatical errors, and corrects the presentation of various trademarks in the specification. Support for the substitute specification may be found throughout the specification as filed and in the original claims. Clean and marked-up versions of the substitute specification are submitted, with changes shown.

The substitute specification contains no new matter. Corrections of typographical and grammatical errors are believed to be self-evident from a review of the marked-up specification, and therefore Applicants limit their comments on the changes made in the substitute specification to the following:

At paragraph 1, claims for priority have been made as required by 37 C.F.R. § 1.52.

At paragraph 2, the sequence listing has been incorporated by reference as required by 37 C.F.R. § 1.52.

At paragraph 13, the text has been amended to clarify that the protein has a molecular weight of 60 kD under reducing conditions, and molecular weights of 60 kD *and* 120 kD under non-reducing conditions. Support for this amendment may be found at page 20, lines 3-8, and at page 44, lines 3-11, in the specification as originally filed, *e.g.*, at current paragraphs 66 and 137.

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[14] See Footnote 1, *supra*.

At paragraph 16, the text has been amended to clarify that the IMR-90 cells are human fetal lung fibroblast cells. Support for this amendment may be found at page 15, lines 3-11, in the specification as originally filed, *e.g.*, at current paragraph 48.

At paragraphs 17-31, the Description of the Figures has been moved (from following paragraph 45) to precede the Detailed Description as suggested in 37 C.F.R. § 1.77, and sequence identifiers have been added to the description of Figures 9-12. Support for the addition of the sequence identifiers may be found in the specification, sequence listing, and figures as originally filed.

At paragraphs 145, 147, 148, 151, 153, 156 and 158, the amino acid numbers used when referring to SEQ ID NO: 5 have been corrected to match the current sequence listing in this application. In the original sequence listing filed in parent application 08/915,004, several amino acid sequences (including SEQ ID NO: 5) were numbered with positive and negative numbers, where the negative numbers referred to amino acids not present in the mature peptides (*e.g.*, signal peptide amino acids had negative numbers). Subsequent to the filing of the parent application, the sequence listing was brought into compliance with the current 37 C.F.R., and accordingly all amino acid sequences are now numbered only with positive numbers. The present amendment now results in the specification matching the sequence listing. Support for these amendments may be found in the specification as originally filed, the sequence listing as originally filed in the parent application, and in the current sequence listing.

At paragraph 255, the number of the Table has been corrected (it was a duplicate number) to reflect its position in the sequence of Tables in the specification.

At paragraphs 261-263, information regarding deposits made under the Budapest Treaty has been added to the specification. Support for the addition of this information may be found in the Statements on the Availability of Deposited Material and Deposit Receipts attached hereto as Appendix A. These Statements were originally submitted in related application 09/338,063 which also claims priority to application number 08/915,004 (the grand-parent of this application).

At paragraph 266, Applicants have re-written the deposit information and provided the current name of the international depository in which the accession resides. Support for this amendment may be found at page 86, lines 14-25, in the specification as originally filed and in the Statements on the Availability of Deposited Material and Deposit Receipts.

At paragraph 267, Applicants have included the text "What is Claimed:". Although this substitute specification does not include the actual claims, Applicants desire to include the text of this heading herein.

Reiterated Request Under 37 C.F.R. § 1.607 For Interference With Patent 6,284,740

As reflected in the chart provided on pages 5-8 of the Preliminary Amendment and Request for Interference filed September 3, 2002 in parent Application No. 10/232,858, claims 32-34 (previously numbered claims 38-43 in the parent applicaiton) correspond substantially to claims found in issued U.S. Patent No. 6,284,740 ('740). Applicants reiterate their request to have an interference declared between these claims and U.S. Patent No. 6,284,740.

CONCLUSION

Prompt and favorable consideration of this Amendment is respectfully requested. Should the Examiner have any questions regarding the above-identified application, he is invited to contact the undersigned at the number indicated below.

Respectfully submitted,

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SUBSTITUTE SPECIFICATION

NOVEL PROTEINS AND METHODS FOR PRODUCING THE PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is a continuation of United States Application Serial Number 10/232,858, filed September 3, 2002, which is a continuation of United States Application Serial Number 08/915,004, filed August 20, 1997, which is a continuation-in-part of international application PCT/JP96/00374, filed February 20, 1996, which claims priority to Japanese applications JP/054977, filed February 20, 1995 and JP/207508, filed July 21, 1995.

INCORPORATION OF SEQUENCE LISTING

[0002] Herein incorporated by reference is the Sequence Listing, which has been submitted on paper and on diskette as a file named "SubSeq16991016.txt" which is 136,653 bytes in size (measured in MS-DOS), and which was created on January 22, 2004.

FIELD OF THE INVENTION ~~Field of the invention~~

[0003] This invention relates to a novel protein, osteoclastogenesis inhibitory factor (OCIF), and methods for producing the protein.

BACKGROUND OF THE INVENTION ~~Background of the invention~~

[0004] Human bones are always remodelling by the repeated process of resorption and reconstitution. ~~In the process, o~~Osteoblasts and osteoclasts are considered to be the cells mainly responsible for bone formation and bone resorption, respectively. A typical example of a disease caused by ~~the progression of~~ abnormal bone metabolism is osteoporosis. ~~The disease~~Osteoporosis is known to ~~be provoked by the condition in which~~ develop when bone

resorption by osteoclasts exceeds bone formation by osteoblasts, but the mechanism of osteoporosis has not yet been completely elucidated. Osteoporosis causes ~~pain in the~~ bone pain and makes ~~the~~ bones fragile, leading to fracture, particularly in elderly patients. ~~Since osteoporosis increases the number of bedridden old people, it~~ Osteoporosis has therefore become a social issue with the increasing number of ~~old~~ elderly people in the population. Therefore, ~~efficacious~~ effective drugs for the treatment of the disease are expected to be developed. Bone mass reduction caused by ~~the~~ abnormal bone metabolism is thought to be prevented by inhibiting bone resorption, improving bone formation, or improving the ~~balanced~~ balance of bone metabolism.

[0005] Bone formation is ~~expected to be~~ promoted by stimulating growth, differentiation, or activation of osteoblasts. Many cytokines ~~are~~ reportedly ~~to~~ stimulate growth or ~~differentation~~ differentiation of osteoblasts, i.e. fibroblast growth factor (FGF) (Rodan S.B. et al., Endocrinology vol. 121, p1917, 1987), insulin-like growth factor-I (IGF-I) (Hock J.M. et al., Endocrinology vol. 122, p254, 1988), insulin-like growth factor-II (IGF-II) (McCarthy T. et al., Endocrinology vol. 124, p301, 1989), Activin A (Centrella M. et al., ~~Mol,~~ Mol. Cell[[,]] Biol. vol. 11, p250, 1991), Vasculotropin (Varonique M. et al., Biochem. Biophys. Res. Commun. vol. 199, p380, 1994), and bone morphogenetic protein (BMP) (Yamaguchi, A. et al., J. Cell Biol. vol. 113, p682, 1991, Sampath T.K. et al., J. Biol. Chem. vol. 267, p20532, 1992, and Knutsen R. et al., Biochem. Biophys. Res. Commun. vol. 194, p1352, 1993).

[0006] On the other hand, cytokines which inhibit[[s]] differentiation and/or maturation of osteoclasts ~~have been paid attention and~~ have also been intensively studied. Transforming growth factor-β (Chenu C. et al., Proc. Natl. Acad. Sci. USA, vol. 85, p5683, 1988) and interleukin-4 (Kasano K. et al., Bone-Miner., vol. 21, p179, 1993) ~~are found to~~ inhibit the differentiation of osteoclasts. Calcitonin (Bone-Miner., vol. 17, p347, 1992), ~~M~~macrophage colony-stimulating factor (Hattersley G. et al., J. Cell. Physiol. vol. 137, p199, 1988), interleukin-4 (Watanabe, K. et al., Biochem. Biophys. Res. Commun. vol. 172, p1035, 1990), and interferon-γ (Gowen M. et al., J. Bone Miner. Res., vol. 1, p469, 1986) ~~are found to~~ inhibit bone resorption by osteoclasts.

[0007] These cytokines are expected to be ~~efficacious~~ effective drugs for improving bone mass reduction by stimulating bone formation and/or by inhibiting bone resorption. ~~The~~ ~~c~~Cytokines

such as insulin-like growth factor-I and bone morphogenetic proteins ~~are now~~ have been investigated in clinical trials for their ~~effects in treatment of~~ effectiveness for treating patients with bone diseases. Calcitonin is already used ~~as a drug to care~~ for osteoporosis and to diminish pain in osteoporosis patients.

[0008] Examples of drugs now clinically utilized for the treatment of bone diseases and for shortening the treatment period are dihydroxyvitamine D_3, vitamin K_2, calcitonin and its derivatives, hormones such as estradiol, ipriflavon, and calcium preparations. However, these drugs do not provide satisfactory therapeutic effects, and novel drug substances ~~have been~~ are expected to be developed. ~~As mentioned,~~Since bone metabolism is ~~controlled~~manifest in the balance between bone resorption and bone formation [[. Therefore]], cytokines which inhibit osteoclast differentiation and/or maturation are expected to be developed as drugs for the treatment of bone diseases such as osteoporosis.

SUMMARY OF THE INVENTION ~~Disclosure of Invention~~

[0009] ~~This invention was initiated from the view point described above.~~ The purpose of this invention is to offer both a novel factor, termed osteoclastogenesis inhibitory factor (OCIF), and a procedure to produce the factor efficiently.

[0010] The inventors have intensively searched for osteoclastogenesis inhibitory factors in human embryonic ~~fibloblast~~ fibroblast IMR-90 (ATCC CCL186) conditioned medium and have found a novel osteoclastogenesis inhibitory factor (OCIF) which inhibits differentiation and/or maturation of osteoclasts.

[0011] The inventors have established a method for accumulating the protein to a high concentration by culturing IMR-90 cells ~~using~~ on alumina ceramic pieces, which function as ~~the~~ cell adherence matrices.

[0012] The inventors have also established an efficient method for isolating the protein, OCIF, from the IMR-90 conditioned medium using the following sequential column chromatography[[,]] : ion-exchange, heparin affinity, cibacron-blue affinity, and reverse phase.

[0013] ~~The inventors, based on~~ After determining the amino acid sequence of the purified natural OCIF, ~~successfully cloned~~ a cDNA encoding this protein was successfully cloned. ~~The~~

~~inventors established also a~~ A procedure ~~to produce~~ for producing this protein ~~which inhibits differentiation of osteoclasts~~ was also established. ~~This~~ The invention concerns a protein which is produced by human lung fibroblast cells, has a molecular weight[[s in]] by SDS-PAGE of 60 ~~KD~~ kD ~~in the~~ under reducing conditions and molecular weights of 60 kD and 120 ~~KD~~ kD under ~~the~~ non-reducing conditions, and has affinity for both cation-exchange resins and heparin[[,]]. The protein's ability to ~~reduces its activity to~~ inhibit the differentiation and maturation of osteoclasts is reduced when ~~if~~ treated for 10 minutes at ~~70 °C~~ 70°C or for 30 minutes at ~~56 °C~~ 56°C, and ~~lose~~ its ~~activity~~ ability to inhibit differentiation and maturation of osteoclasts ~~by the treatment~~ is lost when treated for 10 minutes at ~~90 °C~~ 90°C. The amino acid sequence of the ~~protein~~ OCIF protein ~~which is described in~~ of the present invention is clearly different from any ~~of know~~ other factors known to inhibit the ~~inhibiting~~ formation of osteoclasts.

[0014] The invention includes a method ~~to~~ for purifying OCIF protein, comprising [[;]] : (1) culturing human fibroblasts, (2) applying the conditioned medium to a heparin column to obtain the adsorbed fraction, (3) purifying the OCIF protein using a cation-exchange column, (4) purifying the OCIF protein using a heparin affinity column, (5) purifying the OCIF protein using a ~~cibacron~~ Cibacron blue affinity column, and (6) isolating the OCIF protein using reverse-phase column chromatography. Cibacron blue F3GA dye may be coupled to a carrier made of synthetic hydrophilic polymers, for example, to form ~~is an example of materials used to prepare Cibacron blue columns. These~~ columns ~~are~~ conventionally called "blue ~~colomns~~ columns".

[0015] The invention includes a method for ~~accumulating~~ producing ~~the~~ OCIF protein ~~to a~~ in high concentration by culturing human fibroblasts using alumina ceramic pieces as the cell-adherence matrices.

[0016] Moreover, the inventors determined the amino acid sequences of ~~the~~ peptides derived from OCIF, designed the oligonucleotide primers based on these amino acid sequences, and obtained cDNA fragments encoding OCIF from a cDNA library of IMR-90 human fetal lung fibroblast cells. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using an OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. Recombinant OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria.

BRIEF DESCRIPTION OF THE FIGURES

[0017] Figures 1A and 1B show the elution pattern of crude OCIF protein (HILOAD™-Q/FF pass-through fraction; sample 3) from a HILOAD™ -S/HP column.

[0018] Figure 2 shows the elution pattern of crude OCIF protein (heparin-5PW fraction; sample 5) from a blue-5PW column.

[0019] Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.

[0020] Figures 4A and 4B show the SDS-PAGE results of isolated OCIF proteins under reducing or non-reducing conditions. Description of the lanes:

lane 1, 4: molecular weight marker proteins;

lane 2, 5: OCIF protein of peak 6 in Figure 3;

lane 3, 6: OCIF protein of peak 7 in Figure 3.

[0021] Figure 5 shows the elution pattern of peptides (peak 7) obtained by the digestion of pyridyl ethylated OCIF protein digested with lysylendopeptidase, on a reverse-phase column.

[0022] Figure 6 shows the SDS-PAGE results of isolated natural (n) OCIF protein and recombinant (r) OCIF proteins under non-reducing conditions. rOCIF (E) and rOCIF (C) proteins were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 1: molecular weight marker proteins;

lane 2: a monomer type nOCIF protein;

lane 3: a dimer type nOCIF protein;

lane 4: a monomer type rOCIF (E) protein;

lane 5: a dimer type rOCIF (E) protein;

lane 6: a monomer type rOCIF (C) protein;

lane 7: a dimer type rOCIF (C) protein.

[0023] Figure 7 shows the SDS-PAGE results of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF (E) and rOCIF (C) were produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 8: molecular weight marker proteins;

lane 9: a monomer type nOCIF protein;

lane 10: a dimer type nOCIF protein;

lane 11: a monomer type rOCIF (E) protein;

lane 12: a dimer type rOCIF (E) protein;

lane 13: a monomer type rOCIF (C) protein;

lane 14: a dimer type rOCIF (C) protein.

[0024] Figure 8 shows the SDS-PAGE results of isolated natural (n) OCIF proteins and recombinant (r) OCIF proteins from which N-linked sugar chains were removed under reducing conditions. rOCIF (E) and rOCIF (C) are rOCIF proteins produced by 293/EBNA cells and by CHO cells, respectively. Description of the lanes:

lane 15: molecular weight marker proteins;

lane 16: a monomer type nOCIF protein;

lane 17: a dimer type nOCIF protein;

lane 18: a monomer type rOCIF (E) protein;

lane 19: a dimer type rOCIF (E) protein;

lane 20: a monomer type rOCIF (C) protein;

lane 21: a dimer type rOCIF (C) protein.

[0025] Figure 9 shows a comparison of OCIF (SEQ ID NO: 5) and OCIF2 (SEQ ID NO: 9) amino acid sequences.

[0026] Figure 10 shows a comparison of OCIF (SEQ ID NO: 5) and OCIF3 (SEQ ID NO: 11) amino acid sequences.

[0027] Figure 11 shows a comparison of OCIF (SEQ ID NO: 5) and OCIF4 (SEQ ID NO: 13) amino acid sequences.

[0028] Figure 12 shows a comparison of OCIF (SEQ ID NO: 5) and OCIF5 (SEQ ID NO: 15) amino acid sequences.

[0029] Figure 13 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.

[0030] Figure 14 shows a standard curve determining OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.

[0031] Figure 15 shows the effect of rOCIF protein on model rats with osteoporosis.

~~Detailed description of the invention~~ DETAILED DESCRIPTION OF THE INVENTION

[0032] The OCIF protein of the present invention can be isolated from human fibroblast conditioned medium with high yield. The procedure to isolate OCIF is based on ordinary techniques for purifying proteins from biomaterials, in accordance with the physical and chemical properties of OCIF protein. For example, concentrating procedures include[[s]] ordinary biochemical techniques such as ultrafiltration, ~~lyophylization~~ lyophilization, and dialysis. Purifying procedures include[[s]] combinations of several chromatographic techniques for purifying proteins such as ion-exchange column chromatography, affinity column chromatography, gel filtration column chromatography, hydrophobic column chromatography, reverse phase column chromatography, and preparative gel electrophoresis. The human fibroblasts used for the production of ~~the~~ OCIF protein ~~is~~ are preferably IMR-90 cells. A method for producing ~~the~~ IMR-90 conditioned medium is preferably a process comprising[[,]] adhering human embryonic fibroblast IMR-90 cells to alumina ceramic pieces in roller-bottles[[, using]] in DMEM medium supplemented with 5% ~~new born~~ newborn calf serum ~~for the cell culture~~, and cultivating the cells in roller-bottles for 7 to 10 days by stand cultivation. CHAPS (3-[(3-~~cholamid opropyl~~ cholamidopropyl)-dimethylammonio]-[[l]] 1 -propanesulfonate) is ~~prefarably~~ preferably added to the buffer as a detergent in the protein purification procedure. ~~steps of OCIF protein.~~

[0033] The OCIF protein of the instant invention can be obtained initially ~~obtained~~ as a basic heparin binding ~~basic~~ OCIF fraction by applying the culture medium to a heparin column (~~Heparin-Sepharose~~ Heparin-SEPHAROSE™ CL-6B, Pharmacia), eluting with 10 mM Tris-HCl buffer, pH 7.5, containing 2 M NaCl, and ~~then by~~ applying the OCIF fraction to a Q anion-exchange column (~~Hiload~~ HILOAD™-Q/FF, Pharmacia), and collecting the non-adsorbed fraction. OCIF protein can be purified by subjecting the obtained OCIF fraction to purification on an ~~a~~ S cation-exchange column (~~Hiload~~ HILOAD™-S/FF, Pharmacia), a heparin column (Heparin-5PW, TOSOH), ~~Cibacrone~~ a Cibacron Blue column (Blue-5PW, TOSOH), and a reverse-phase column (BU-300 C4, Perkin Elmer) ~~and the material is defined by the previously described properties~~.

[0034] The present invention relates to a method of cloning cDNA encoding the OCIF protein based on the amino acid sequence of natural OCIF and a method for [[of]] obtaining recombinant OCIF protein ~~that inhibits differentiation and/or maturation of osteoclasts~~. The OCIF protein is purified according to the method described in the present invention and is treated with endopeptidase (for example, lysylendopeptidase). The amino acid sequences of the peptides produced by the digestion are determined and the mixture of oligonucleotides that can encode each internal amino acid sequence ~~was~~ is ~~systhesized~~ synthesized. The OCIF cDNA fragment is obtained by PCR (preferably RT-PCR, reverse transcriptase PCR) using the oligonucleotide mixtures described above as primers. The full length OCIF cDNA encoding the OCIF protein is cloned from a cDNA library using ~~the obtained~~ an OCIF DNA fragment as a probe. The OCIF cDNA containing the entire coding region is inserted into an expression vector. ~~The~~ ~~r~~Recombinant OCIF can be produced by expressing the OCIF cDNA, containing the entire coding region, in mammalian cells or bacteria.[[ss]]

[0035] The present invention relates to the novel proteins OCIF2, OCIF3, OCIF4, and OCIF5 that are variants of OCIF and have the activity described above. These OCIF variants are obtained from the cDNA library constructed with IMR-90 poly(A) + RNA ~~by hybridization~~ using the OCIF cDNA fragment as a hybridization probe. Each of the OCIF variant cDNAs containing the entire coding region is inserted into an expression vector. Each recombinant OCIF variant protein can be produced by expressing each of the OCIF variant cDNAs, containing the entire coding region, in ~~the~~ conventional hosts. Each recombinant OCIF variant

protein can be purified according to the method described in this invention. Each recombinant OCIF variant protein has ~~an~~ the ability to inhibit osteoclastogenesis.

[0036] The present invention further includes OCIF mutants. They are substitution mutants comprising the replacement of one cysteine residue, possibly involved in dimer formation, with a serine residue[[,]] ~~and~~ or various deletion mutants of OCIF. Substitutions or deletions are introduced into the OCIF cDNA using polymerase chain reaction (PCR) or ~~by~~ restriction enzyme digestion. Each of these mutated OCIF cDNAs is inserted into a vector ~~containing~~ having an appropriate promoter for gene expression. The resultant expression vector for each of the OCIF mutants is ~~transfected~~ introduced into eukaryotic cells such as mammalian cells. Each of OCIF mutants can be obtained and purified from the conditioned media of the transfected cells.

[0037] The present invention provides polyclonal antibodies and a method to quantitatively determine OCIF concentration using these polyclonal antibodies.

[0038] ~~As antigens (immunogens), n~~Natural OCIF obtained from IMR-90 conditioned medium, recombinant OCIF produced by such hosts as microorganisms and eukaryotes using OCIF cDNA, synthetic peptides ~~designed~~ based on the amino acid sequence of OCIF, or peptides obtained from OCIF by partial digestion can be used as antigens. Anti-OCIF polyclonal antibodies are obtained by immunizing appropriate mammals with the antigens, in combination with adjuvants ~~for immunization~~ if necessary, and purifying the antibodies from the serum by ~~the~~ ordinary purification methods. ~~The a~~Anti-OCIF polyclonal antibodies which are labelled with ~~rasioisotopes~~ radioisotopes or enzymes can be used in radio-immunoassay (RIA) systems or enzyme-immunoassay~~iminunoassa~~y (EIA) systems. ~~By u~~Using these assay systems, the concentration[[s]] of OCIF in biological materials such as blood~~and~~, ascites and ~~cells culture~~ cell-culture medium can be easily determined.

~~The antibodies in the present invention can be used in radio-immunoassay (RIA) or enzyme immunoassay (EIA). By using these assay systems, the concentration of OCIF in biological materials such as blood and ascites can be easily determined.~~

[0039] The present invention provides novel monoclonal antibodies and a method ~~to~~ for quantitatively ~~determine~~ determining OCIF concentration using these monoclonal antibodies.

[0040] Anti-OCIF monoclonal antibodies can be produced by ~~the~~ conventional methods using OCIF as an antigen. Native OCIF obtained from the culture medium of IMR-90 cells and recombinant OCIF produced by such hosts as microorganisms and eukaryotes ~~using~~ transfected with OCIF cDNA can be used as antigens. Alternatively, ~~synthesized~~ synthetic peptides ~~designed~~ based on the amino acid sequence of OCIF and peptides obtained from OCIF by partial digestion can be also used as antigens. Immunized lymphocytes obtained by ~~immunization of~~ immunizing mammals such as mice or rats with the antigen or by an in vitro immunization method were fused with mammalian myeloma cells ~~of mammals~~ to obtain hybridomas. The hybridoma clones secreting antibodies[[y]] which recognize[[s]] OCIF ~~from the hybridomas obtained by the cell fusion~~ were selected and cultured to obtain [[. T]]the desired antibodies. ~~can be obtained by cell culture of the selected hybridoma clones. In preparation of hybridoma, small animals such as mice or rats are generally used for immunization.~~ ~~To immunize~~ For immunizations, OCIF is suitably diluted with a saline solution (0.15 M ~~NACl~~ NaCl), and is intravenously or intraperitoneally administered with an adjuvant to animals ~~for~~ 2-5 times every 2-20 days. The immunized animal was killed three days after the final immunization, the spleen was removed ~~taken out~~ and the splenocytes were used as immunized B lymphocytes.

[0041] Mouse myeloma cell lines useful for cell fusion with ~~the~~ immunized B lymphocytes include, for example, p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, FO, p3x63 Ag8.653, and S194 cells. The [[R]]rat cell line [[R-21O]] R-210 ~~cell line~~ may also be used. Alternatively [[H]]human B lymphocytes ~~are~~ immunized by an in vitro immunization method ~~and~~ are fused with human myeloma cells ~~line~~ or EB virus transformed human B lymphocytes ~~which are used as a parent cell line for cell fusion,~~ to produce human type antibodies[[y]].

[0042] Cell fusion of ~~the~~ immunized B lymphocytes and myeloma ~~cell line~~ cells is carried out principally by ~~the~~ conventional methods. For example, the method of Koehler G. et al[[,.]]. (Nature 256, 495-497, 1975) is generally used[[, and also]]. Alternatively, an electric pulse method can be used ~~applied to cell fusion~~. The immunized B lymphocytes and transformed B cells are mixed at conventional ratios and a cell culture medium without FBS containing polyethylene glycol is generally used to fuse the cells ~~for cell fusion~~. The ~~B lymphocytes fused with myeloma cell lines~~ fusions products are cultured in HAT selection medium containing FBS to select hybridomas.

[0043] ~~For screening of hybridoma producing anti-OCIF antibody,~~ [[a]]An EIA, plaque assay, Ouchterlony, or agglutination assay can be ~~principally~~ used to screen for hybridomas producing anti-OCIF antibodies. ~~Among them,~~ EIA is a simple ~~and easy to operate~~ assay which is easy to perform with sufficient accuracy and is therefore generally used. [[By EIA using purified OCIF, t]]The desired antibody can be selected easily and accurately using EIA and purified OCIF. [[Thus obtained h]]Hybridomas obtained thereby can be cultured by ~~the~~ conventional methods of cell culture and frozen for stock if necessary. The antibody can be produced by culturing hybridoma cells using ~~the~~ ordinary cell culture methods or by transplanting hybridoma cells intraperitoneally into live animals. The antibody can be purified by ~~the~~ ordinary purification methods such as salt precipitation, gel filtration, and affinity chromatography. The ~~obtained~~ antibody obtained specifically reacts with OCIF and can be used ~~for determination of~~ to determine OCIF concentration and ~~for purification of~~ to purify OCIF protein. The antibodies of the present invention recognize epitopes of OCIF and have high affinity ~~to~~ for OCIF. Therefore, they can be used for the construction of EIA. [[By (using) t]]This assay system[[,]] is useful for determining the concentration of OCIF in biological materials such as blood and ascites ~~can be easily determined.~~

[0044] The present invention provides agents[[, used for treating bone diseases that contain]] containing OCIF as an effective ingredient, that are useful for treating bone diseases [[provided by the present invention]]. Rats were subjected to denervation of the left forelimb. Test compounds were administered daily after surgery for 14 days. After 2 weeks of treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical strength by the three point bending method. OCIF improved the mechanical strength of bone in a dose dependent manner.

[0045] The OCIF protein of the invention is useful as a pharmaceutical ingredient[[s]] for treating or improving decreased bone mass in bone diseases such as osteoporosis, ~~bone diseases such as~~ rheumatism, osteoarthritis, and abnormal bone metabolism in multiple myeloma. OCIF protein is also useful as an antigen ~~to establish~~ in the immunological diagnosis of bone diseases. Pharmaceutical preparations containing the OCIF protein as an active ingredient[[s]] are formulated and can be orally or parenterally administered. The preparation contains the OCIF protein of the present invention as an ~~efficacious~~ effective ingredient and is safely administered to humans and animals. Examples of ~~the~~ pharmaceutical preparations include compositions for

injection or intravenous drip, suppositories, nasal preparations, sublingual preparations, and tapes for percutaneous absorption. The pharmaceutical preparation for injection can be prepared by mixing ~~the~~ a pharmacologically ~~efficacious~~ effective amount of OCIF protein and a pharmaceutically acceptable carrier[[s]]. The carriers are vehicles and/or activators, e.g. amino acids, saccharides, cellulose derivatives, and other organic and inorganic compounds, which are generally added to active ingredients. When the OCIF protein is mixed with the vehicles and/or activators for injection, pH adjusters, buffers, stabilizers, solubilizing agents, etc. can be added by conventional methods, if necessary.

~~Brief description of the figures~~

~~Figure 1 shows the elution pattern of crude OCIF protein (HILOAD-Q/FF pass-through fraction; sample 3) from a HILOAD-S/HP column.~~

~~Figure 2 shows the elution pattern of crude OCIF protein (heparin-5PW fraction ; sample 5) from a blue-5PW column.~~

~~Figure 3 shows the elution pattern of OCIF protein (blue-5PW fraction 49 to 50) from a reverse-phase column.~~

~~Figure 4 shows the SDS-PAGE of isolated OCIF proteins under reducing conditions or non-reducing conditions.~~

~~Description of the lanes,~~

~~lane 1,4 ; molecular weight marker proteins~~

~~lane 2, 5 ; OCIF protein of peak 6 in figure 3~~

~~lane 3,6 ; OCIF protein of peak 7 in figure 3~~

~~Figure 5 shows the elution pattern of peptides obtained by the digestion of pyridyl-ethylated OCIF protein digested with lysylendopeptidase, on a reverse-phase column.~~

~~Figure 6 shows the SDS-PAGE of isolated natural(n) OCIF protein and recombinant(r) OCIF proteins under non-reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.~~

~~Description of the lanes,~~

~~lane 1 ; molecular weight marker proteins~~

~~lane 2 ; a monomer type nOCIF protein~~

~~lane 3 ; a dimer type nOCIF protein~~

~~lane 4 ; a monomer type rOCIF(E) protein~~

~~lane 5 ; a dimer type rOCIF(E) protein~~

~~lane 6 ; a monomer type rOCIF(C) protein~~

~~lane 7 ; a dimer type rOCIF(C) protein~~

~~Figure 7 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant (r) OCIF proteins under reducing conditions. rOCIF(E) and rOCIF(C) were produced in 293/EBNA cells and in CHO cells, respectively.~~

~~Description of the lanes,~~

~~lane 8 ; molecular weight marker proteins~~

~~lane 9 ; a monomer type nOCIF protein~~

~~lane 10 ; a dimer type nOCIF protein~~

~~lane 11 ; a monomer type rOCIF(E) protein~~

~~lane 12 ; a dimer type rOCIF(E) protein~~

~~lane 13 ; a monomer type rOCIF(C) protein~~

~~lane 14 ; a dimer type rOCIF(C) protein~~

~~Figure 8 shows the SDS-PAGE of isolated natural(n) OCIF proteins and recombinant(r) OCIF proteins from which N-linked sugar chains were removed under reducing conditions. rOCIF(E) and rOCIF(C) are rOCIF protein produced in 293/EBNA cells and in CHO cells, respectively.~~

~~Description of the lanes,~~

~~lane 15 ; molecular weight marker proteins~~

~~lane 16 ; a monomer type nOCIF protein~~

~~lane 17 ; a dimer type nOCIF protein~~

~~lane 18 ; a monomer type rOCIF(E) protein~~

~~lane 19 ; a dimer type rOCIF(E) protein~~

~~lane 20 ; a monomer type rOCIF(C) protein~~

~~lane 21 ; a dimer type rOCIF(C) protein~~

~~Figure 9 shows comparison of amino acid sequences between OCIF and OCIF2.~~

~~Figure 10 shows comparison of amino acid sequences between OCIF and OCIF3.~~

~~Figure 11 shows comparison of amino acid sequencôs between OCIF and OCIF4.~~

~~Figure 12 shows comparison of amino acid sequences between OCIF and OCIF5.~~

~~Figure 13 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF polyclonal antibodies.~~

~~Figure 14 shows standard curve for determination of OCIF protein concentration by an EIA employing anti-OCIF monoclonal antibodies.~~

~~Figure 15 shows the effect of rOCIF protein on osteoporosis.~~

Best Mode for Carrying Out the Invention

[0046] The present invention will be further explained by the following examples, ~~however,~~ though the scope of the invention is not restricted thereto ~~to the examples~~.

EXAMPLE 1

[0047] Preparation of a conditioned medium of human fibroblast IMR-90

[0048] Human fetal lung fibroblast IMR-90 (ATCC-CCL186) cells were cultured on alumina ceramic pieces (80 g) (alumina: 99.5%, manufactured by Toshiba Ceramic K.K.) in DMEM medium (manufactured by Gibco BRL Co.) supplemented with 5% CS and ~~10mM~~ 10 mM HEPES buffer (500 ml/roller bottle) at 37°C ~~under~~ in the presence of 5% ~~$C0_2$~~ CO_2 for 7 to 10 days using 60 roller bottles (490 cm^2 , 110 x ~~171mm~~ 171 mm, manufactured by Corning Co.) in static culture. The conditioned medium was harvested and a fresh medium was added to the roller bottles. About ~~30L~~ 30 L of IMR-90 conditioned medium per batch culture was obtained. The conditioned medium was designated as sample 1.

EXAMPLE 2

[0049] Assay method for osteoclast development inhibitory activity

[0050] Osteoclast development inhibitory activity was assayed by measuring tartrate-resistant acid phosphatase (TRAP) activity according to the methods of M. Kumegawa et[[.]] al. (Protein Nucleic Acid [[ ]]Enzyme, vol. 34 p999, 1989) and N. Takahashi et al. (~~Endocrynology~~ Endocrinology, vol. 122, p1373, 1988) with modifications. Briefly, bone marrow cells obtained from a 17 day-old mouse were suspended in ~~α-MEM~~ α-MEM™ (manufactured by GIBCO BRL Co.) containing 10% FBS, ~~$2x 10^{-8}M$~~ 2×10^{-8} M of activated vitamin D_3 and a ~~each~~ test sample and were inoculated into each well of a 96-well plate at a cell density of ~~$3x10^5$~~ 3×10^5 cells/0.2 ml/well. The plates were incubated for 7 days at 37°C in humidified ~~5%CO_2~~ 5% CO_2. Cultures were ~~further continued~~ maintained by replacing 0.16 ml of old medium with the same volume of fresh medium on day 3 and day 5 after ~~starting~~ cultivation began. On day 7, ~~after washing~~ the plates were washed with phosphate buffered saline, and the cells were fixed with ethanol/acetone (1:1) for 1 min. at room temperature[[, and then]]. [[o]]Osteoclast development was tested by determining ~~for~~ acid phosphatase activity using a kit (Acid Phosphatase, Leucocyte, Catalog No. 387-A, manufactured by Sigma Co.). A decrease in the number of TRAP positive cells was taken as an indication of OCIF activity.

EXAMPLE 3

[0051] Purification of OCIF

[0052] i) Heparin ~~Sepharose~~ SEPHAROSE™ CL-6B column chromatography

[0053] ~~The 90L~~ 90 L of IMR-90 conditioned medium (sample 1) was ~~filtrated with~~ filtered using a 0.22 μ membrane filter (hydrophilic ~~Milidisk~~MILLIDISK™[[,]] 2000 [[CM]]cm^2, Millipore Co.), and was divided into three 30 liter portions. Each portion ~~(30 L)~~ was applied to a heparin ~~Sepharose~~ SEPHAROSE™ CL-6B column (5 x 4.1 cm, Pharmacia Co.) equilibrated with ~~10mM~~ 10 mM Tris-HC1 containing ~~0.3M~~ 0.3 M NaCl, pH 7.5. After washing the column with ~~10mM~~ 10 mM Tris-HC1, pH 7.5 at a flow rate of 500 ml/hr., the heparin ~~Sepharose~~

SEPHAROSE™ CL-6B adsorbent protein fraction was eluted with ~~10mM~~ 10 mM Tris-HC1, pH 7.5, containing ~~2M~~ 2 M NaCl. The fraction was designated sample 2.

[0054] ii) ~~Hiload~~ HILOAD™-Q/FF column chromatography

[0055] The heparin ~~Sepharose~~ SEPHAROSE™-adsorbent fraction (sample 2) was dialyzed against ~~10mM~~ 10 mM Tris-HC1, pH 7.5, supplemented with CHAPS to a final concentration of 0.1%, incubated at ~~4 °C~~ 4°C overnight and divided into two portions. Each portion was then applied to an anion-exchange column (~~Hiload~~ HILOAD™-Q/FF, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with ~~50mM~~ 50 mM Tris-HC1, 0.1% CHAPS, pH 7.5 to obtain a non-adsorbent fraction (1000 ml). The fraction was designated sample 3.

[0056] iii) ~~Hiload~~ HILOAD™-S/HP column chromatography

[0057] The ~~Hiload~~ HILOAD™-Q non-adsorbent fraction (sample 3) was applied to a cation-exchange column (~~Hiload~~ HILOAD™-S/HP, 2.6 x 10 cm, Pharmacia Co.) which was equilibrated with 50 mM Tris-HCl, ~~0.1 %~~ 0.1% CHAPS, pH ~~7. 5~~ 7.5. After washing the column with 50 mM Tris-HC1, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted with a linear gradient from 0 to 1 M NaCl at a flow rate of 8 ml/min for 100 min. and fractions (12 ml) were collected. ~~Each~~ Every ten fractions from numbers 1 to 40 were pooled to form one portion. ~~Each~~ 100 ~~µ l~~ µL each of the four portions was tested for OCIF activity. OCIF activity was observed in fractions ~~from~~ 11 to 30 (as shown in Figures 1A and 1B). ~~The~~ Fractions ~~from~~ 21 to 30, which had higher specific activity, were ~~collected~~ pooled and ~~was~~ designated sample 4.

[0058] iv) Heparin-5PW affinity column chromatography

[0059] One hundred and twenty ml of ~~Hiload~~ HILOAD™-S fractions ~~from~~ 21 to 30 (sample 4) was diluted with 240 ml of 50 mM ~~Tris-HCl~~ Tris-HCl, 0.1% CHAPS, pH ~~7. 5~~ 7.5, and applied to a heparin-5PW affinity column (0.8 x 7.5 cm, Tosoh Co.) which was equilibrated with ~~50mM~~ 50 mM Tris-HC1, 0.1% CHAPS, pH 7.5. After washing the column with ~~50mM~~ 50 mM Tris-HC1, 0.1% CHAPS, pH ~~7. 5~~ 7.5, the adsorbed protein was eluted with a linear gradient from 0 to ~~2M~~ 2 M NaCl at a flow rate of ~~0.5ml/min~~ 0.5 ml/min for 60 min. and fractions (0.5 ml) were collected. Fifty ~~µ l~~ µL ~~was~~ were removed from each fraction to test for OCIF activity. The active fractions, eluted with 0.7 to ~~1.3M~~ 1.3 M NaCl ~~was~~ were pooled and ~~was~~ designated ~~as~~ sample 5.

[0060] v) Blue 5PW affinity column chromatography

[0061] Ten ml of sample 5 ~~was~~ were diluted with 190 ml of ~~50mM~~ 50 mM Tris-HC1, 0.1% CHAPS, pH 7.5 and applied to a blue-5PW affinity column, (~~0. 5 x5~~ 0.5 x 5 cm, Tosoh Co.) which was equilibrated with ~~50mM~~ 50 mM Tris-HC1, ~~0.1 %~~ 0.1% CHAPS, pH 7.5. After washing the column with ~~50mM~~ 50 mM Tris-HC1, 0.1% CHAPS, ~~pH7.5~~ pH 7.5, the adsorbed protein was eluted with a 30 ml linear gradient from 0 to ~~2M~~ 2 M NaCl at a flow rate of 0.5 ml/min. and fractions (0.5 ml) were collected. Using 25 ~~μ1~~ μL of each fraction, OCIF activity was evaluated. [[The f]]Fractions ~~numbered~~ 49 to 70, eluted with ~~1.0-1.6M~~ 1.0-1.6 M NaCl, had OCIF activity.

[0062] vi) Reverse phase column chromatography

[0063] The blue 5PW fraction obtained by collecting fractions ~~from~~ 49 and 50 was acidified with 10 ~~μ1~~ μL of 25% TFA and applied to a reverse phase C4 column (BU-300, ~~2.1x220mm~~ 2.1 x 220 mm, manufactured by Perkin-Elmer) which was equilibrated with 0.1% of TFA and 25% ~~of~~ acetonitrile. The adsorbed protein was eluted with a linear gradient from 25 to 55% acetonitrile at a flow rate of 0.2 ml/min. for 60 min., and each protein peak was collected (Fig. 3). One hundred ~~μ1~~μL of each peak fraction was tested for OCIF activity, and peaks 6 and ~~the peak~~ 7 had OCIF activity. The result was shown in Table 1.

Table 1

~~OCIF activity eluted from reverse phase C4 column~~

Sample	Dilution			
	1/40	1/120	1/360	1/1080
Peak 6	++	++	+	-
Peak 7	++	+	-	-

[++ means OCIF activity inhibiting osteoclast development more than 80%, + means OCIF activity inhibiting osteoclast development between 30% and 80%, and - means no OCIF activity.]

EXAMPLE 4

[0064] Molecular weight of OCIF protein

[0065] The two protein peaks with OCIF activity (peaks 6 and 7) ~~with OCIF activity~~ were subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Briefly, ~~20μ l~~ 20 μL of each peak fraction was concentrated under vacuum and dissolved in 1.5 ~~μ l~~ μL of ~~10mM~~ 10 mM]]Tris-HC1]] Tris-HCl, pH 8, ~~1mM~~ 1 mM EDTA, 2.5% SDS, ~~0. 01%~~ 0.01% bromophenol blue, and incubated at 37°C overnight under non-reducing conditions or under reducing conditions (with 5% of 2-mercaptoethanol). Each 1.0 ~~μ l~~ μL of sample was then analyzed by SDS-polyacrylamide gel electrophoresis with a gradient gel of 10-15% acrylamide (Pharmacia Co.) and an electrophoresis-device (Fast System, Pharmacia Co.). The following molecular weight marker proteins were used to calculate molecular weight: ~~phosphorylise~~ phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (~~20. 0~~ 20.0 kD), and lactalbumin (~~14. 4~~ 14.4 kD). After electrophoresis, protein bands were visualized by silver stain using Phast Silver Stain Kit. The results are shown in Fig. 4.

[0066] A protein band with an apparent molecular weight of 60 ~~KD~~ kD was detected in the peak 6 ~~protein~~ sample under both reducing and non-reducing conditions. A protein band with an apparent 60 ~~KD~~ kD was detected under reducing conditions and a protein band with an apparent 120 ~~KD~~ kD was detected under non-reducing conditions in the peak 7 ~~protein~~ sample. Therefore, the protein of peak 7 was considered to be a homodimer of the protein of peak 6.

EXAMPLE 5

[0067] Thermostability of OCIF

[0068] Twenty ~~μ l~~ μL of sample from the blue-5PW fractions 51 and 52 was diluted to 30 ~~μ l~~ μL with 10 mM phosphate buffered saline, pH 7.2, and incubated for 10 min. at 70°C or 90°C, or for 30 min. at 56°C. The heat-treated samples were tested for OCIF activity. The results ~~were~~ are shown in Table 2.

Table 2

Thermostability of OCIF

Sample	Dilution		
	1/300	1/900	1/2700
[[u]]Untreated	++	+	-
70°C, 10 min	+	-	-
56°C, 30 min	+	-	-
90°C, 10 min	-	-	-

[++ means OCIF activity inhibiting osteoclast development more than 80%, ~~+means~~ + means OCIF activity inhibiting osteoclast development between 30% and 80%, and - means no OCIF activity.]

EXAMPLE 6

[0069] Internal amino acid sequence of OCIF protein

[0070] Each 2 fractions (1 ml) from fractions ~~No.~~ 51 to 70 of the blue-5PW fractions [[was]] were acidified with 10 ~~μ-l~~ μL of 25% TFA, and ~~was~~ applied to a reverse phase C4 column (BU-300, ~~2.1x220mm~~ 2.1 x 220 mm, manufactured by Perkin-Elmer Co.) equilibrated with 25% acetonitrile containing 0.1% TFA. The adsorbed protein was eluted with a 12 ml linear gradient of 25 to 55% acetonitrile at a flow rate of 0.2 ml/min, and the protein fractions corresponding to peaks 6 and 7 were collected, respectively. The protein ~~of~~ from each peak was applied to a protein sequencer (~~PROCISE~~ PROCISE™ 494, Perkin-Elmer Co.). However, the N-terminal sequence of the protein of each peak could not be analyzed. Therefore, ~~N-terminal~~ the N-terminus of the protein of each peak was considered to be blocked. [[So, i]]Internal amino acid sequences of these proteins were therefore analyzed.

[0071] The protein ~~of~~ from peak 6 or 7 purified by C4-HPLC, was concentrated by centrifugation and pyridilethylated under reducing conditions. Briefly, ~~50μl~~ 50 μL of 0.5 M ~~Tris-HCl~~ Tris-HCl, pH 8.5, containing 100 μg of dithiothreitol, ~~10mM~~ 10 mM EDTA, 7 M ~~guanidine-HCl~~ guanidine-HCl, and 1% CHAPS was added to each of the samples, and the

mixtures were ~~was~~ incubated overnight in the dark at room temperature. Each ~~the~~ mixture was acidified with 25% TFA (a final concentration 0.1%) and was applied to a reverse[[d]] phase C4 column (BU300, ~~2.1x30mm~~ 2.1 x 30 mm, Perkin-Elmer Co.) equilibrated with 20% acetonitrile containing ~~0.1 %~~ 0.1% TFA. The pyridil-ethylated OCIF protein was eluted with a 9 ml linear gradient from 20 to 50% acetonitrile at a flow rate of 0.3 ml/min, and each protein peak was collected. The ~~pyridil-ethyrated~~ pyridil-ethylated OCIF protein was concentrated under vacuum and dissolved in ~~25µl~~ 25 µL of 0.1 M Tris-HC1, pH 9, containing 8 M Urea, and ~~0.1 % Tween~~ 0.1% TWEEN™ 80. ~~Seventy three~~ Seventy-three ~~µl~~µL of 0.1 M ~~Tris-HCl~~ Tris-HCl, pH 9, and ~~0. 02~~ 0.02 µg of lysyl endopeptidase (Wako Pure Chemical, Japan) were added to the tube, and incubated at ~~37 °C~~ 37°C for 15 hours. Each digest was acidified with 1 ~~µl~~µL of 25% TFA and was applied to a reverse phase C8 column (RP-300, ~~2.1x220mm~~ 2.1 x 220 mm, Perkin-Elmer Co.) equilibrated with 0.1% TFA.

[0072] The peptide fragments were eluted from the column with a linear gradient ~~from~~ of 0 to 50% acetonitrile at a flow rate of 0.2 ml/min for 70 min., and each peptide peak was collected. Each peptide fragment (P1 - P3) was applied to the protein sequencer. The sequences of the peptides are shown in ~~Sequence Numbers~~ SEQ ID NOs: 1-3, respectively.

EXAMPLE 7

[0073] Determination of the nucleotide sequence of OCIF cDNA

[0074] i) Isolation of poly(A) + RNA from IMR-90 cells

[0075] About 10 µg of poly(A) + RNA was isolated from ~~$1x10^8$~~ 1×10^8 cells of IMR-90 using a ~~Fast Track~~ FASTTRACK™ mRNA isolation kit (Invitrogen) according to the manufacturer's instructions.

[0076] ii) Preparation of mixed primers

[0077] The following two mixed primers were synthesized based on the amino acid sequences of two peptides (peptide P2 and peptide P3, ~~sequence numbers~~ SEQ ID NOs: 2 and 3, respectively). All the oligonucleotides in the mixed primers No. 2F (SEQ ID NO: 107) can code for the amino acid sequence from the sixth residue, glutamine (Gln) to the twelfth residue, leucine (Leu), in peptide P2. All the oligonucleotides in the mixed primers No. 3R (SEQ ID NO: 108) can code for the amino acid sequence from the sixth residue, histidine (His), to the twelfth

residue, lysine (Lys), in peptide P3. The sequences of the mixed primers No. 2F and No. 3R were shown in Table 3.

Table 3

No. 2F (SEQ ID NO: 107)

```
5'-CAAGAACAAA CTTTTCAATT-3'
       G  G  G  C  C  GC
                   A
                   G
```

No. 3R (SEQ ID NO: 108)

```
5'-TTTATACATT GTAAAAGAAT G-3'
    C G     C  G  GCTG
            A    C
            G    T
```

[0078] iii) Amplification of an OCIF cDNA fragment by PCR (Polymerase chain reaction)

[0079] First strand cDNA was generated using a ~~Superscript~~ SUPERSCRIPT™ II cDNA synthesis kit 23 (Gibco BRL) and 1 μg of poly(A) + RNA obtained in ~~the example~~ EXAMPLE 7-i), according to the manufacturer's instructions. The DNA fragment encoding OCIF was obtained by PCR using cDNA template and the primers shown in EXAMPLE 7-ii).

PCR was performed ~~with the~~ using the following conditions [[as follows;]]:

~~10X~~ 10x Ex Taq Buffer (Takara Shuzo)	5	~~μl~~ μL
~~2.5mM~~ 2.5 mM solution of dNTPs	4	~~μl~~ μL
cDNA solution	1	~~μl~~ μL
Ex Taq (Takara Shuzo)	0.25	~~μl~~ μL
sterile distilled water	29.75	~~μl~~ μL
40 ~~uM~~ μM solution of primers No. 2F	5	~~μl~~ μL
40 ~~uM~~ μM solution of primers No. 3R	5	~~μl~~ μL

[0080] The components of the reaction were mixed in a microcentrifuge tube. An initial denaturation step at 95°C for 3 min was followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extention at 70°C for ~~2min.~~ 2 min. After the amplification, a final extention step was performed at 70°C for ~~5min.~~ 5 min. The sizes of the PCR products were determined on a ~~1.5 %~~ 1.5% agarose gel electrophoresis. ~~About~~ An approximately 400 bp OCIF DNA fragment was obtained.

EXAMPLE 8

[0081] Cloning of the OCIF cDNA fragment amplified by PCR and determination of its DNA sequence

[0082] The OCIF cDNA fragment amplified by PCR in EXAMPLE ~~7iii~~ 7-iii) was inserted ~~in~~ into the plasmid ~~pBluescript~~ pBLUESCRIPT II ~~SK~~ SK™ using a DNA ligation kit ver. 2 (Takara Shuzo) according to the method of Marchuk, D. et al. (Nucleic Acids Res., vol 19, ~~P1154~~ p1154, 1991). *E. coli* strain DH5 α (Gibco BRL) was transformed with the ligation mixture. The transformants were grown and a plasmid containing the OCIF cDNA (about 400 bp) was purified using ~~the~~ commonly used methods. This plasmid was called pBSOCIF. The sequence of the OCIF cDNA in pBSOCIF was determined using a ~~Taq Dye Deoxy Terminater Cycle Sequencing~~ TAQ DYE DEOXY TERMINATER CYCLE SEQUENCING™ kit (Perkin Elmer). The size of the OCIF cDNA is 397 bp. The OCIF cDNA encodes an amino acid sequence containing 132 residues. The amino acid sequences of the internal peptides (peptide P2 and peptide P3, ~~sequence number~~ SEQ ID NOs: 2 and 3, respectively) that were used to design

the primers were found at ~~N- or C-terminal side~~ the amino or carboxyl terminus of the 132 ~~in the~~ amino acid sequence ~~of the 132 amino acid polypeptide~~ predicted by the 397 bp OCIF cDNA. In addition, the amino acid sequence of the internal peptide P1 (~~sequence number~~ SEQ ID NO: 1) was also found in the predicted amino acid sequence of OCIF. These data show that the 397 bp OCIF ~~EDNA~~cDNA is a portion of the full length OCIF cDNA.

EXAMPLE 9

[0083] Preparation of the DNA probe

[0084] The 397 bp OCIF cDNA was prepared according to the conditions described in EXAMPLE ~~7iii~~ 7-iii). The OCIF cDNA was subjected to a preparative agarose gel electrophoresis. The OCIF cDNA was purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN), labeled with [α^{32}P]dCTP using Megaprime DNA labeling system (Amersham) and used to select a phage containing the full length OCIF cDNA.

EXAMPLE 10

[0085] Preparation of the cDNA library

[0086] cDNA was generated using a Great Lengths cDNA synthesis kit (Clontech), oligo (dT) ~~primer,[α^{32}P]dCTP~~ primer, [α^{32}P]dCTP and 2.5 μg of poly(A) + RNA obtained in ~~the example~~ EXAMPLE 7-i), according to the manufacturer's[[-]] instructions. An EcoRI-SalI-NotI adaptor was ligated to the cDNA. The cDNA was separated from free adaptor DNA and unincorporated free [α^{32}P]dCTP. The purified cDNA was precipitated with ethanol and dissolved in 10 ~~μl~~ μL of TE buffer (10 ~~mMTris-HCl~~ mM Tris-HC1 (~~pH8.0~~ pH 8.0), 1 mM EDTA). The cDNA comprising ~~with~~ the adaptor was ~~inserted~~ ligated into λZAP ~~EXPRESS~~ EXPRESS™ vector (Stratagene) at the EcoRI site. The recombinant λZAP ~~EXPRESS~~ EXPRESS™ phage DNA containing the cDNA was in vitro packaged using a ~~Gigapack gold~~ GIGAPACK™ gold # II packaging extract (Stratagene) ~~and~~ yielding a recombinant λZAP ~~EXPRESS~~ EXPRESS™ phage library ~~was prepared~~.

EXAMPLE 11

[0087] Screening of recombinant phage

[0088] Recombinant phages obtained in EXAMPLE 10 were ~~infected to~~ used to infect *E. coli* strain, XL1-Blue MRF' (Stratagene) at ~~37 °C~~ 37°C for 15 min. The infected ~~E.coli~~ *E. coli* cells were added to NZY medium containing ~~0. 7 %~~ 0.7% agar at 50°C and plated onto ~~on the~~ NZY agar plates. After the plates were incubated at ~~37 °C~~ 37°C overnight, ~~Hybond N~~ HYBOND^{TM} N (Amersham) membranes were placed on the surface of the plates containing plaques. The membranes were denatured in ~~the~~ alkali solution, neutralized, and washed in ~~2xSSC~~ 2 x SSC according to ~~the~~ standard methods ~~protocol~~. The phage DNA was immobilized onto ~~on~~ the membranes using UV ~~Crosslink~~ CROSSLINK^{TM} (Stratagene). The membranes were incubated in ~~the~~ hybridization buffer (Amersham) containing 100 μg/ml salmon sperm DNA at 65°C for 4 hours and then incubated at ~~65 °C~~ 65°C overnight in the same buffer containing ~~2x10~~^{5} 2 x 10^5 cpm/ml of denatured OCIF DNA probe. The membranes were washed twice with ~~2xSSC~~ 2 x SSC and twice with a solution containing ~~0. 1xSSC~~ 0.1 x SSC and ~~0. 1 %~~ 0.1% SDS at ~~65 °C~~ 65°C for 10 min each time. The positive clones were purified by repeating the screening twice. The purified ~~λ ZAP EXPRESS~~ λZAP EXPRESS^{TM} phage clone containing a DNA insert of about 1.6 kb ~~insert~~ was used in the experiments described below. This phage was called ~~λOCIF~~ λ OCIF. The purified ~~λOCIF~~ λ OCIF was used to infect ~~and the infected into~~ *E. coli* strain XL-1 blue MRF' (Stratagene) according to the protocol in the ~~λ ZAP EXPRESS~~ λZAP EXPRESS^{TM} cloning kit (Stratagene). The culture broth of infected XL-1 blue MRF' was prepared. Purified ~~lOCIF~~ λ OCIF and ~~ExAssist~~ EXASSIST^{TM} helper phage (Stratagene) were coinfected into *E. coli* strain XL-1 blue MRF', according to the protocol supplied with the kit. The culture broth of the coinfected XL-1 blue MRF' was added to a culture of *E. coli* strain XLOR (Stratagene) to transform them. Thus we obtained a Kanamycin-resistant transformant harboring a plasmid designated pBKOCIF which is a pBKCMV (Stratagene) vector containing the 1.6 kb insert fragment.

[0089] The transformant including the plasmid containing about 1.6 kb OCIF cDNA was obtained by ~~picking up~~ lifting the [[k]]Kanamycin-resistant colonies. The plasmid was called pBKOCIF. The transformant has been deposited ~~to~~ in the National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology as "FERM BP-5267"

as pBK/01F10. A national deposit (Accession number, FERM P-14998) was transferred[[-]] to the international deposit, on October 25, 1995 according to the Budapest treaty. The transformant pBK/01F10 was grown and the plasmid ~~PBKOCIF~~ pBKOCIF was purified according to ~~the~~ standard methods ~~protocol~~.

EXAMPLE 12

[0090] Determination of the nucleotide sequence of OCIF cDNA containing the full coding region.

[0091] The nucleotide sequence of OCIF cDNA obtained in EXAMPLE 11 was determined using a ~~Taq Dye Deoxy Terminater Cycle Sequencing~~ TAQ DYE DEOXY TERMINATER CYCLE SEQUENCING™ kit (Perkin Elmer). The primers used were T3, T7 (Stratagene) and synthetic primers designed according to the OCIF cDNA sequence. The sequences of these primers are shown in ~~sequence numbers~~ SEQ ID NOs: 16 to 29. The nucleotide sequence of the OCIF cDNA is shown in ~~sequence number~~ SEQ ID NO: 6 and the amino acid sequence predicted by the cDNA sequence is shown in ~~sequence number~~ SEQ ID NO: 5.

EXAMPLE 13

[0092] Production of recombinant OCIF by 293/EBNA cells

[0093] ~~1)~~ i) Construction of the plasmid for expressing OCIF cDNA

[0094] pBKOCIF, containing about 1.6 kb OCIF cDNA, [[-]]was prepared as described in EXAMPLE 11[[,]] and digested with restriction enzymes BamHI and XhoI. The OCIF cDNA insert was cut out, ~~separated~~ isolated by an agarose gel electrophoresis and purified using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The purified OCIF cDNA insert was ligated ~~using DNA ligation kit ver. 2 (Takara Shuzo)~~ into the expression vector pCEP4 (Invitrogen) using DNA ligation kit ver. 2 (Takara Shuzo) digested with restriction enzymes[[,]] ~~BamHI~~ BamHI and XhoI. *E. coli* strain DH5 α (Gibco BRL) was transformed with the ~~ligation-mixture~~ ligation mixture.

[0095] The transformants were grown and the plasmid containing the OCIF cDNA (about 1.6 kb) was purified using a ~~QIAGEN~~ QIAGEN™ column (QIAGEN). The expression plasmid pCEPOCIF was precipitated with ethanol and dissolved in sterile distilled water ~~was used~~ for use in the experiments described below.

[0096] ii) Transient expression of OCIF cDNA and analysis of ~~the~~ OCIF biological activity

[0097] Recombinant OCIF was produced using the expression plasmid pCEPOCIF (prepared in EXAMPLE 13-i) according to the method described below. ~~8x~~ 8 x 10^5 cells of 293/EBNA (Invitrogen) were inoculated into each well of ~~the~~ a 6-well plate using IMDM containing ~~10 %~~ 10% fetal ~~calf~~ bovine serum (FBS; Gibco BRL). After the cells were incubated for 24 hours, the culture medium was removed and the cells were washed with serum free IMDM. The expression plasmid pCEPOCIF and lipofectamine (Gibco BRL) were diluted with ~~OPTI-MEM~~ OPTI-MEM™ (Gibco BRL), ~~and were~~ mixed, and added to the cells in each well according to the ~~manufacture's~~ manufacturer's instructions. Three ~~μ g~~ μg of pCEPOCIF and 12 ~~μl~~ μL of lipofectamine ~~was~~ were used for each transfection. After the cells were incubated with pCEPOCIF and lipofectamine for 38 hours, the medium was replaced with 1 ml of ~~OPTI-MEM~~ OPTI-MEM™. After ~~the transfected cells were incubated~~ incubation for 30 hours, the conditioned medium was harvested and used for the biological assay. The biological activity of OCIF was analyzed according to the method described below. Bone marrow cells obtained from 17 day old mice[[, 17 days old,]] were suspended in ~~α-MEM~~ α-MEM™ (manufactured by GIBCO BRL Co.) containing 10% FBS, ~~$2x10^{-8}$M~~ 1 x 10^{-8}M activated vitamin D_3 and ~~each~~ a test sample, and were inoculated and cultured for 7 days at 37°C in humidified ~~5%$C0_2$~~ 5% CO_2 as described in EXAMPLE 2. During incubation, 160 ~~μl~~ μL of old medium in each well was replaced with the same volume of the fresh medium containing test sample diluted with ~~$1x10^{-8}$M~~ 1 x 10^{-8} M of activated vitamin D_3 and ~~α-MEM~~ α-MEM™ containing FBS on day 3 and day 5. On day 7, after washing the wells with phosphate buffered saline, cells were fixed with ethanol/acetone (1:1) for 1 min. and osteoclast development was tested using an acid phosphatase activity measuring kit (Acid Phosphatase, Leucocyte, Catalog ~~No.387A~~ No. 387A, Sigma Co.). ~~The~~ A decrease ~~of~~ in the number of TRAP positive cells was taken as an OCIF activity. [[As a result, t]]The conditioned medium showed the same OCIF activity as natural OCIF protein from IMR-90 conditioned medium (Table 4).

Table 4

OCIF activity of 293/EBNA conditioned medium.

Cultured Cell	Dilution						
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
OCIF expression vector transfected	++	++	++	++	++	+	-
vector transfected	-	-	-	-	-	-	-
untreated	-	-	-	-	-	-	-

[++; OCIF activity inhibiting osteoclast development more than 80%, +; OCIF activity inhibiting osteoclast development between 30% and 80%, and -; no OCIF activity.]

[0098] iii) Isolation of recombinant OCIF protein from 293/EBNA-conditioned medium

[0099] 293/EBNA-conditioned medium (1.8 ~~l~~L) obtained by cultivating the cells described in ~~example 13ii~~ EXAMPLE 13-ii) was supplemented with ~~0. 1 %~~ 0.1% ~~of~~ CHAPS and filtrated ~~with~~ using a 0.22 μm membrane filter (~~Steribecs~~ Sterivex GS, Millipore ~~Co.). The~~ Co.). The conditioned medium was applied to a 50 ml ~~of a~~ heparin ~~Sepharose~~ SEPHAROSE™ CL-6B column (~~2. 6~~ 2.6 x 10 cm, Pharmacia Co.) equilibrated with ~~10mM Tris-HCl~~ 10 mM Tris-HCl, pH ~~7.5. After~~ 7.5. After washing the column with ~~10mM Tris-HCl~~ 10 mM Tris-HCl, pH ~~7. 5~~ 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8 ml fractions [[(8 ml)]] were collected. Using 150 ~~μl~~ μL of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An OCIF active 112 ml fraction [[(112 ml)]], eluted with approximately 0.6 to 1.2 M NaCl, was obtained.

[0100] One hundred twelve ml of the active fraction was diluted to 1000 ml with 10 mM Tris-HCl, 0.1% CHAPS, pH ~~7. 5~~ 7.5, and applied to a heparin affinity column (heparin-5PW, 0.8 x 7.5 cm, Tosoh Co.) equilibrated with ~~10mm~~ 10 mM Tris-HCl, 0.1% CHAPS, pH 7.5. After

washing the column with ~~10mM~~ 10 mM Tris-HC1, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of ~~0. 5ml~~ 0.5 ml/min for 60 min. and 0.5 ml fractions [[(0.5 ml)]] were collected. Four ~~μl~~ μL of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. ~~On SDS-PAGE under reducing conditions, a~~ A single band of rOCIF protein with an apparent molecular weight of 60 ~~KD~~ kD was detected in fractions from 30 to 32 by SDS-PAGE under reducing conditions. [[, under non-reducing conditions, b]]Bands of rOCIF protein with apparent molecular weights of 60 ~~KD~~ kD and 120 ~~KD~~ kD were also detected in fractions from 30 to 32 under non-reducing conditions. The isolated rOCIF from fractions ~~from~~ 30 to 32 was designated as recombinant OCIF derived from 293/EBNA (rOCIF(E)). 1.5 ml of the rOCIF(E) (535 ~~μ g~~ μg/ml) was obtained when determined by the method of Lowry, using bovine serum albumin as a standard protein.

EXAMPLE 14

[0101] Production of recombinant OCIF using CHO cells

[0102] i) Construction of the plasmid for expressing OCIF

[0103] pBKOCIF containing about 1.6 kb OCIF cDNA was prepared as described in EXAMPLE 11, and digested with restriction enzymes SalI and EcoRV. About 1.4 kb OCIF cDNA insert was separated by agarose gel electrophoresis and purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The expression vector, pcDL-SR α 296 (Molecular and Cellular Biology, vol 8, p466-472, 1988) was digested with restriction enzymes PstI and KpnI. About 3.4 kb of the expression vector fragment was cut out, separated by agarose gel electrophoresis and purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The ends of the purified OCIF cDNA insert and the expression vector fragment were blunted using a DNA blunting kit (Takara Shuzo). [[The-purified]] The purified OCIF cDNA insert and the expression vector fragment were ligated using a DNA ligation kit ver. 2 (Takara Shuzo). *E. coli* strain DH5a α (Gibco BRL) was transformed with the ligation mixture. A ~~The~~ transformant containing the OCIF expression plasmid, pSR ~~α OCIF~~ αOCIF was obtained.

[0104] ii) Preparation of the expression plasmid

[0105] The transformant containing the OCIF expression plasmid, pSR ~~α OCIF~~ αOCIF prepared in ~~the example~~ EXAMPLE 13-i) and the transformant containing the mouse DHFR expression plasmid, pBAdDSV shown in ~~W092/01053~~ WO 92/01053 were grown according to ~~the~~ standard methods. Both plasmids were purified by alkali treatment, polyethylene glycol precipitation, and cesium chloride density gradient ultra centrifugation according to the method of Maniatis et al. (*Molecular* ~~cloning~~ *Cloning*, 2nd edition).

[0106] iii) Adaptation of [[CHOdhFr-]] CHOdhFr⁻ cells to the protein free medium

[0107] [[CHOdhFr-]] CHOdhFr⁻ cells (ATCC, CRL 9096) were cultured in IMDM containing 10% fetal ~~calf~~ bovine serum. The cells were adapted to ~~EXCELL~~ EXCELL™ 301 (JRH ~~Bioscieence~~ Bioscience) and then adapted to ~~EXCELL~~ EXCELL™ PF CHO (JRH ~~Bioscieence~~ Bioscience) according to the ~~manufacture's~~ manufacturer's instructions.

[0108] iv) Transfection of the OCIF expression plasmid, and the mouse DHFR expression plasmid, into [[CHOdhFr-]] CHOdhFr⁻cells.

[0109] [[CHOdhFr-]] CHOdhFr⁻ cells prepared in EXAMPLE 14-iii) were transfected by electroporation with ~~pSRαOCIF~~ pSR αOCIF and pBAdDSV prepared in EXAMPLE 14-ii). Two hundred μg of pSR ~~α OCIF~~ αOCIF and 20 μg of pBAdDSV were dissolved under sterile conditions in 0.8 ml of IMDM (Gibco BRL) containing 10% fetal ~~calf~~ bovine serum. ~~$2x10^7$ cells of~~ [[CHOdhFr-]] CHOdhFr⁻ cells ($2x10^7$) were suspended in 0.8 ml of this medium. The cell suspension was ~~transfered~~ transferred to a cuvette (Bio Rad) and the cells were transfected by electroporation using a ~~gene pulser~~ GENE PULSER™(Bio Rad) under the conditions of 360 V and 960 μF. The suspension of electroporated cells was transferred to T-flasks (Sumitomo Bakelite) containing 10 ml of ~~EXCELL~~ EXCELL™ PF-CHO, and incubated in the ~~$C0_2$~~ CO_2 incubator for 2 days. The transfected cells were then inoculated into each well of a 96 well plate (Sumitomo Bakelite) at a density of 5000 cells/well and cultured for about 2 weeks. The transformants expressing DHFR are selected since ~~EX-CELL~~ EXCELL™ PF-CHO does not contain nucleotides and the parental cell line [[CHOdhFr-]] CHOdhFr⁻ can not grow in this medium. Most of the transformants expressing DHFR express OCIF since the OCIF expression plasmid was used ten times as much as the mouse ~~DHFR/expression plasmid~~ DHFR expression

plasmid. The transformants whose conditioned medium had high OCIF activity were selected from among the transformants expressing DHFR according to the method described in EXAMPLE 2. The transformants that express large amounts of OCIF were cloned by the limiting dilution method. The clones whose conditioned medium had high OCIF activity were selected as described above and a transformant expressing large amounts of OCIF named, 5561, was obtained.

[0110] v) Production of recombinant OCIF

[0111] To produce recombinant OCIF (rOCIF), clone 5561 was inoculated into a 3 L spinner flask with ~~EX-CELL~~ EXCELL™ 301 medium (3 ~~l~~ L) ~~in a 3 l spiner flask was inoculated with the clone (5561)~~ at a cell density of ~~1x10^5~~ 1 x 10^5 cells/ml. The 5561 cells were cultured in a ~~spiner~~ spinner flask at 37°C for 4 to 5 days. When the concentration of the 5561 cells reached ~~1x10^6~~ 1 x 10^6 cells/ml, about 2.7 ~~l~~ L of the conditioned medium was harvested. Then about 2.7 ~~l~~ L of ~~EX-CELL~~ EXCELL™ 301 was added to the ~~spiner~~ spinner flask and the 5561 cells were ~~was~~ cultured repeatedly.

[0112] About 20 ~~l~~ L of the conditioned medium was harvested using the three ~~spiner~~ spinner flasks.

[0113] vi) Isolation of recombinant OCIF protein from CHO cell[[s]]-conditioned medium

[0114] [[CHOcells-conditioned]] CHO cell-conditioned medium (1.0 [[l]] L) described in EXAMPLE 14-v) was supplemented with 1.0 g CHAPS and filtrated with a 0.22 μm membrane filter (~~Steribecks~~ Sterivex-GS, Millipore Co.). The conditioned medium was applied to a heparin ~~Sepharose~~ SEPHAROSE™-FF column (2.6 x 10 cm, Pharmacia Co.) equilibrated with 10 mM Tris-HC1, pH 7.5. After washing the column with 10 mM ~~Tris-HCI~~ Tris-HCl, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 2 M NaCl at a flow rate of 4 ml/min for 100 min. and 8 ml fractions [[(8 ml)]] were collected. Using ~~150 μl~~ 150 μL of each fraction, OCIF activity was assayed according to the method described in EXAMPLE 2. An active fraction (112 ml) eluted [[with.approximately]] with approximately 0.6 to 1.2 M NaCl was obtained.

[0115] The 112 ml ~~of~~ active fraction was diluted to 1200 ml with 10 mM Tris-HC1, 0.1% CHAPS, pH 7.5, and applied to an affinity column (Blue-5PW, ~~0. 5~~ 0.5 x 5.0 cm, Tosoh Co.)

equilibrated with 10 mM Tris-HC1, 0.1% CHAPS, pH 7.5. After washing the column with 10 mM Tris-HC1, 0.1% CHAPS, pH 7.5, the adsorbed protein was eluted from the column with a linear gradient from 0 to 3 M NaCl at a flow rate of ~~0.5ml~~ 0.5 ml/min for 60 min. and fractions (~~0. 5~~ 0.5 ml) were collected. Four ~~µl~~ µL of each fraction were subjected to SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions as described in EXAMPLE 4. [[On SDS-PAGE under reducing conditions, a]] A single band of rOCIF protein with an apparent molecular weight of 60 ~~KD~~ kD was detected in fractions 30 to 38 using SDS-PAGE under reducing conditions. [, under non-reducing conditions, b]]Bands of rOCIF protein with apparent molecular weights of 60 ~~KD~~ kD and 120 kD ~~KD~~ using SDS-PAGE under non-reducing conditions were also detected in fractions 30 to 38. The isolated rOCIF fraction, from fractions 30 to 38, was designated as purified recombinant OCIF derived from CHO cells (rOCIF(C)). 4.5 ml of the rOCIF(C) (113 µg/ml) was obtained, as ~~when~~ determined by the method of Lowry using bovine serum albumin as a standard protein.

EXAMPLE 15

[0116] Determination of N-terminal amino acid sequence of rOCIFs

[0117] Three ~~Each 3 µ g~~ µg of the isolated rOCIF(E) and rOCIF(C) ~~was~~ were adsorbed to polyvinylidene difluoride (PVDF) membranes with ~~Prospin~~ PROSPIN™ (PERKIN ELMER Co.). The membranes were washed with 20% ethanol and the N-terminal amino acid sequences of the adsorbed proteins were analyzed by protein sequencer (~~PROCISE~~ PROCISE™ 492, PERKIN ELMER Co.). The determined N-terminal amino acid sequence is shown in ~~sequence~~ SEQ ID NO: 7.

[0118] The N-terminal amino acid of rOCIF(E) and rOCIF(C) was ~~the 22th amino acid of gluatmine from Met as translation starting point~~ glutamic acid located at position 22 from Met of the translation start site, as shown in ~~sequence number~~ SEQ ID NO: 5. The 21 amino acids from Met to Gln were identified as a signal peptide. The N-terminal amino acid sequence of OCIF isolated from IMR-90 conditioned medium ~~was undetectable~~ could not be determined. Accordingly, the N-terminal ~~glutamine~~ glutamic acid of OCIF may be blocked by ~~converting from~~ the conversion of ~~glutamine~~ glutamic acid to pyroglutamine within ~~culturing~~ cell culture or ~~purifying~~ purification steps.

EXAMPLE 16

[0119] Biological activity of ~~recombinant(r)~~ recombinant (r) OCIF and ~~natural(n)~~ natural (n) OCIF

[0120] i) Inhibition of vitamin D_3 induced osteoclast formation in murine bone marrow cells

[0121] Each of the rOCIF(E) and nOCIF samples ~~was~~ were diluted with ~~α-MEM~~ α-MEM™ (GIBCO BRL Co.) containing 10% FBS and ~~2x10^-8^~~ 2×10^{-8} M of activated vitamin D_3 (a final concentration of 250 ng/ml). Each sample was serially diluted with the same medium, and 100 ~~µl~~µL of each diluted sample was added to each well [[in]] of a 96-well plate[[s]]. Bone marrow cells obtained from 17 day old mice[[, 17 days-old,]] were inoculated at a cell density of ~~$3x10^5$~~ 3×10^5 cells/100 ~~µl~~µL/well into each well [[in]] of a 96-well plate[[s]] and cultured for 7 days at 37°C in humidified ~~$5\%CO_2$~~ 5% CO_2. On day 7, the cells were fixed and stained with an acid phosphatase ~~mesuring~~ measuring kit (Acid Phosphatase, Leucocyte, ~~No387-A~~ No. 387-A, Sigma) according to the method described in EXAMPLE 2. ~~The~~ A decrease ~~of~~ in acid phosphatase activity (TRAP) was taken as an indication of OCIF activity. ~~The~~ A decrease ~~of~~ in acid phosphatase-positive cells was evaluated by solubilizing the pigment of dye and measuring absorbance. ~~In detail~~ Briefly, 100 ~~µl~~µL of a mixture of 0.1 N NaOH and dimethylsulfoxide (1:1) was added to each well and the well was vibrated to solubilize the dye. After solubilizing the dye completely, an absorbance of each well was measured at 590 nm, subtracting the absorbance at 490 nm using a microplate reader (~~Immunoreader~~ IMMUNOREADER™ NJ-2000, InterMed). The microplate reader was adjusted to 0 absorbance using a well with monolayered bone marrow cells which ~~was~~ were cultured in the medium without activated vitamin D_3. ~~The~~ A decrease ~~of~~ in TRAP activity was expressed as a percentage of the control absorbance value (=100%) ~~of the solubilized dye from~~ (measured for wells with bone marrow cells cultured in the absence of OCIF). The results are shown in Table 5.

Table 5

Inhibition of vitamin ~~D3-induced~~ D_3-induced osteoclast formation from murine bone marrow cells

OCIF concentration(ng/ml)	250	125	63	31	16	0
rOCIF(E)	0	0	3	62	80	100
nOCIF	0	0	27	27	75	100 (%)

[0122] Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner ~~in the~~ at concentrations of 16 ng/ml or ~~higher~~ greater.

[0123] ii) Inhibition of vitamin ~~D3-induced~~ D_3-induced osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

[0124] The [[E]]effect of OCIF on osteoclast formation induced by Vitamin D_3 in co-cultures of stromal cells and mouse spleen cells was tested according to the method of N. Udagawa et al. (Endocrinology, vol. 125, p1805-1813, 1989). ~~In detail~~ Briefly, samples of each of rOCIF(E), rOCIF(C), and nOCIF ~~sample was~~ were serially diluted with ~~α-MEM~~ α-MEM™ (GIBCO BRL Co.[[ ]]) containing 10% FBS, ~~$2x10^{-8}M$~~ 2×10^{-8} M activated vitamin D_3 and ~~$2x10^{-7}M$~~ 2×10^{-7} M dexamethasone and 100 ~~μl~~ μL of each of the diluted samples was added to each well of 96 well-microwell plates. Murine bone marrow-derived stromal ST2 cells (RIKEN Cell Bank RCB0224) at ~~$5x10^3$~~ 5×10^3 cells per 100~~μl~~ μL of ~~α-MEM~~ α-MEM™ containing 10% FBS and spleen cells from 8 week old ddy mice[[[, 8 weeks-old;]] at ~~$1x10^5$~~ 1×10^5 cells per 100 μl in the same medium, were inoculated into each well of a 96-well plate[[s]] and cultured for 5 days at 37°C in humidified ~~$5\%CO_2$~~ 5% CO_2. On day 5, the cells were fixed and stained ~~with a kit for~~ using an acid phosphatase kit (Acid Phosphatase, Leucocyte, ~~No387-A~~ No. 387-A, Sigma). ~~The~~ A decrease ~~of~~ in acid phosphatase-positive cells was taken as an indication of OCIF activity. ~~Thedecrease~~ The decrease in acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results are shown in Table 6[[;]] (rOCIF(E) and rOCIF(C)) and Table 7[[;]] (rOCIF(E) and ~~nOCIF~~ nOCIF).

Table 6

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

OCIF concentration (ng/ml)	50	25	13	6	0
rOCIF(E)	3	22	83	80	100
rOCIF(C)	13	19	70	96	100 (%)

Table 7

Inhibition of osteoclast formation in co-cultures of stromal cells and mouse spleen cells.

OCIF concentration(ng/ml)	250	63	16	0
rOCIF(E)	7	27	37	100
rOCIF(C)	13	23	40	100 (%)

[0125] nOCIF, rOCIF(E) and rOCIF(C) inhibited osteoclast formation in a dose dependent manner ~~in the~~ at concentrations of 6 - 16 ng/ml or ~~higher~~ greater.

[0126] iii) Inhibition of PTH-induced osteoclast formation in murine bone marrow cells.

[0127] The [[E]]effect of OCIF on osteoclast formation induced by PTH was tested according to the method of N. Takahashi et al. (Endocrinology, vol. 122, p1373-1382, 1988). ~~In detail~~ Briefly, samples of each of ~~the~~ rOCIF(E) and nOCIF (125 ng/ml) were serially diluted with ~~α-MEM~~ α-MEM™ (manufactured by GIBCO BRL Co.) containing 10% FBS and ~~$2x10^{-8}M$~~ 2×10^{-8} M PTH, and ~~100μl~~ 100 μL of each of the diluted samples was added to the wells of 96 well-plates. Bone marrow cells from 17 day old ddy mice[[, 17 day old,]] at a cell density of ~~$3x10^5$~~ 3×10^5 cells per 100 ~~μl~~μL of ~~α-MEM~~ α-MEM™ containing 10% FBS were inoculated into each well [[in]] of a 96-well[[s]] plate and cultured for 5 days at 37°C in humidified ~~5%CO_2~~ 5% CO_2. On day 5, the cells were fixed with ethanol/acetone (1:1) for 1 min. at room

temperature and stained with ~~a kit for~~ an acid phosphatase kit (Acid Phosphatase, Leucocyte, ~~No387-A~~ No. 387-A, Sigma) according to the method described in EXAMPLE 2. ~~The~~ A decrease ~~of~~ in acid phosphatase-positive cells was taken as an indication of OCIF activity. The decrease in acid phosphatase-positive cells was evaluated according to the method described in EXAMPLE 16-i). The results are shown in Table 8.

Table 8

Inhibition of PTH-induced osteoclast formation from murine bone marrow cells.

OCIF concentration(ng/ml)	125	63	31	16	8	0
rOCIF(E)	6	58	58	53	88	100
nOCIF	18	47	53	56	91	100

[0128] nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner ~~in the~~ at concentrations of 16 ng/ml or greater.

[0129] iv) Inhibition of IL-11-induced osteoclast formation

[0130] The [[E]]effect of OCIF on osteoclast formation induced by IL-11 was tested according to the method of T. Tamura et al. (Proc. [[ ]]Natl. Acad. Sci. USA, vol. 90, p11924-11928, 1993). ~~In detail~~ Briefly, samples of each of rOCIF(E) and nOCIF ~~sample was~~ were serially diluted with ~~α-MEM~~ α-MEM™ (GIBCO BRL Co.) containing 10% FBS and 20 ng/ml IL-11 and 100 μl of each ~~the~~ diluted sample was added to each well in a 96-well plate[[s]]. Newborn mouse calvaria-derived pre-adipocyte MC3T3-G2/PA6 cells (RIKEN Cell Bank RCB1127)[[;]] at ~~$5x10^3$~~ 5×10^3 cells per 100 μl of ~~α-MEM~~ α-MEM™ containing 10% FBS, and spleen cells from 8 week old ddy mouse, [[8 weeks-old,;]] at ~~$1x10^5$~~ 1×10^5 cells per 100 ~~μ l~~ μL in the same medium, were inoculated into each well ~~in~~ of a 96-well plate[[s]] and cultured for 5 days at ~~37 °C~~ 37°C in humidified ~~5%CO_2~~ 5% CO_2. On day 5, the cells were fixed and stained with ~~a kit for~~ an acid phosphatase kit (Acid Phosphatase, Leucocyte, ~~No387-A~~ No. 387-A, Sigma). Acid

phosphatase positive cells were counted under a microscope and a decrease of the cell numbers was taken as an indication of OCIF activity. The results are shown in Table 9.

Table 9

OCIF concentration(ng/ml)	500	125	31	7.8	2.0	0.5	0
nOCIF	0	0	1	4	13	49	31
rOCIF(E)	0	0	1	3	10	37	31

[0131] Both nOCIF and rOCIF(E) inhibited osteoclast formation in a dose dependent manner ~~in the~~ at concentrations of 2 ng/ml or ~~higher~~ greater.

[0132] The results shown in Tables 4-8 indicated that OCIF inhibits all the vitamin D_3, PTH, and IL-11-induced osteoclast formations at almost the same doses. Accordingly, OCIF could ~~would~~ be ~~able to be~~ used for treating ~~the treatment of the~~ different types of bone disorders ~~with~~ due to decreased bone mass, ~~which~~ that are caused by different substances ~~which~~ that induce bone resorption.

EXAMPLE 17

[0133] Isolation of monomer-type OCIF and dimer-type OCIF

[0134] Each rOCIF(E) and rOCIF(C) sample containing 100 ~~μ g~~ μg of OCIF protein, was supplemented with 1/100 volume of 25% trifluoro acetic acid and applied to a reverse phase column (~~PROTEIN-RP~~ PROTEIN-RP™, ~~2.0x250~~ 2.0 x 250 mm, YMC Co.) equilibrated with 30% acetonitrile containing 0.1% trifluoro acetic acid. OCIF protein was eluted from the column with a linear gradient from 30 to 55% acetonitrile at a flow rate of 0.2 ml/min for 50 min. and each OCIF peak was collected. ~~Each~~ [[t]]The monomer-type OCIF peak fraction and dimer-type OCIF peak fraction ~~was~~ were each lyophilized[[, respectively]].

EXAMPLE 18

[0135] Determination of the molecular weight of recombinant OCIFs

[0136] Each 1 µg of the isolated monomer-type and dimer-type nOCIF purified using a reverse phase column according to EXAMPLE 3-iv) and each 1 µg of monomer-type and dimer-type rOCIF described in EXAMPLE 17 was concentrated under vacuum[[, respectively]]. Each sample was incubated in the buffer for SDS-PAGE, subjected to SDS-polyacrylamide gel electrophoresis, and protein bands on the gel were stained with silver according to the method described in EXAMPLE 4. Results of electrophoresis under non-reducing conditions and reducing conditions are shown in Figures 6 and ~~Figure~~ 7, respectively.

[0137] A protein band with an apparent molecular weight of 60 ~~KD~~ kD was detected in each monomer-type OCIF sample, and a protein band with an apparent molecular weight of 120 ~~KD~~ kD was detected in each dimer-type OCIF sample ~~in~~ under non-reducing conditions. A protein band with an apparent molecular weight of 60 ~~KD~~ kD was detected in each monomer-type OCIF sample under reducing conditions. Accordingly, the molecular weights of monomer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells and rOCIF from CHO cells were almost the same (~~60kD~~ 60 kD). Molecular weights of dimer-type nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells were also the same (~~120kD~~ 120 kD).

EXAMPLE 19

[0138] Removal of the N-linked ~~Oligosaccharide~~ oligosaccharide chain and [[Mesuring]] measuring the molecular weight of natural and recombinant OCIF

[0139] Each sample containing 5 µg of the isolated monomer-type and dimer-type nOCIF purified using a reverse phase column according to EXAMPLE 3-iv) and each sample containing 5 µg of monomer-type and dimer-type rOCIF described in EXAMPLE 17 were concentrated under ~~vaccum~~ vacuum. Each sample was dissolved in 9.5 ~~µ l~~ µL of 50 mM sodium phosphate buffer, pH 8.6, containing 100 mM 2-mercaptoethanol, supplemented with 0.5 ~~µ l~~ µL of 250 U/ml N-glycanase (Seikagaku kogyo Co.) and incubated for one day at ~~37 °C~~ 37°C. Each sample was supplemented with 10 ~~µ l~~ µL of 20 mM Tris-HC1, pH 8.0 containing 2 mM EDTA, 5% SDS, and ~~0. 02%~~ 0.02% bromo-phenol blue and heated for 5 min at 100 °C. Each 1 ~~µ l~~ µL

of the samples was subjected to SDS-polyacrylamide gel electrophoresis, and protein bands on the gel were stained with silver as described in EXAMPLE 4. The patterns of electrophoresis are shown in Figure 8.

[0140] An apparent molecular weight of each of the deglycosylated nOCIF from IMR-90 cells, rOCIF from CHO cells, and rOCIF from 293/EBNA cells was 40 ~~KD~~ kD under reducing conditions. An apparent molecular weight of each of the untreated nOCIF from IMR-90 cells, rOCIF from 293/EBNA cells, and rOCIF from CHO cells was 60 ~~KD~~ kD under reducing conditions. Accordingly, the results indicate that the OCIF proteins are glycoproteins with N-linked sugar chains.

EXAMPLE 20

[0141] Cloning of OCIF variant cDNAs and determination of their DNA sequences

[0142] The plasmid pBKOCIF, comprising ~~which is inserted~~ OCIF cDNA inserted into plasmid pBKCMV (Stratagene), was obtained ~~from one of some purified phage~~ as in ~~example~~ EXAMPLES 10 and 11. ~~And more~~ Further, during the screening of the cDNA library with the 397 bp OCIF cDNA probe, the transformants containing plasmids whose insert sizes were different from that of pBKOCIF were obtained. These transformants containing the plasmids were grown and the plasmids were purified according to the standard method. The sequence of the insert DNA in each plasmid was determined using a ~~Taq Dye Deoxy Terminater Cycle Sequencing~~ TAQ DYE DEOXY TERMINATER CYCLE SEQUENCING™ kit (Perkin Elmer). The primers used were T3, ~~T7,(Stratagene)~~ T7, (Stratagene) and synthetic primers prepared based on the nucleotide sequence of OCIF cDNA. There are four OCIF variants (OCIF2, 3, 4, and 5) in addition to OCIF. The nucleotide sequence of OCIF2 is shown in ~~the sequence number~~ SEQ ID NO: 8 and the amino acid sequence of ~~OCIF 2~~ OCIF2 predicted by the nucleotide sequence is shown in ~~the sequence number~~ SEQ ID NO: ~~9. The~~ 9. The nucleotide sequence of OCIF3 is shown in ~~the sequence number~~ SEQ ID NO: 10 and the amino acid sequence of OCIF3 predicted by the nucleotide sequence is shown in ~~the sequence number~~ SEQ ID NO: 11. The nucleotide sequence of OCIF4 is shown in ~~the sequence number~~ SEQ ID NO: 12 and the amino acid sequence of OCIF4 predicted by the nucleotide sequence is shown in ~~the sequence number~~ SEQ ID NO: 13. The nucleotide sequence of OCIF5 is shown in ~~the sequence number~~ SEQ ID

NO: 14 and the amino acid sequence of OCIF5 predicted by the nucleotide sequence is shown in ~~the sequence number~~ SEQ ID NO: 15. The structures of OCIF variants are shown in Figures 9 to 12 and are briefly described ~~in brief~~ below.

[0143] OCIF2

[0144] The OCIF2 cDNA has a deletion of 21 bp from guanine at nucleotide number 265 to guanine at nucleotide number 285 in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6).

[0145] Accordingly, OCIF2 has a deletion of 7 amino acids from glutamic acid (Glu) at amino acid number 89 ~~68~~ to glutamine (Gln) at amino acid number 95 ~~74~~ in OCIF (~~sequence number~~ SEQ ID NO: 5).

[0146] OCIF3

[0147] The OCIF3 cDNA has a point mutation at nucleotide number 9 in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6) where cytidine is replaced with guanine. Accordingly, OCIF3 has a mutation ~~and~~ where asparagine (Asn) at amino acid number [[-19]] 3 in OCIF (~~sequence number~~ SEQ ID NO: 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and ~~have~~ has no essential effect on the ~~secreted~~ secretion of OCIF3. OCIF3 cDNA has a deletion of 117 bp from guanine at nucleotide number 872 to cytidine at nucleotide number 988 in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6).

[0148] Accordingly, OCIF3 has a deletion of 39 amino acids from threonine (Thr) at amino acid number 291 ~~270~~ to leucine (Leu) at amino acid number 329 ~~308~~ in OCIF (~~sequence number~~ SEQ ID NO: 5).

[0149] OCIF4

[0150] The OCIF4 cDNA has two point mutations in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6). Cytidine at nucleotide number 9 is replaced with guanine and guanine at nucleotide number 22 is replaced with thymidine in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6).

[0151] Accordingly, OCIF4 has two mutations. Asparagine (Asn) at amino acid number [[-19]] 3 in OCIF (~~sequence number~~ SEQ ID NO: 5) is replaced with lysine (Lys), and alanine (Ala) at amino acid number [[-14]] 8 is replaced with serine (Ser). These mutations seem to be located in the signal sequence and have no essential effect on the secreted OCIF4.

[0152] The OCIF4 cDNA has about 4 kb DNA, comprising ~~which is the~~ intron 2 of the OCIF gene, inserted between nucleotide number 400 and nucleotide number 401 in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6). The open reading frame stops in intron 2.

[0153] Accordingly, OCIF4 has an additional novel amino acid sequence containing 21 amino acids after alanine (Ala) at amino acid number 133 ~~112~~ in OCIF (~~sequence number~~ SEQ. ID No. 5).

[0154] OCIF5

[0155] The OCIF5 cDNA has a point mutation at nucleotide number 9 in the OCIF cDNA (~~sequence number~~ SEQ ID NO: 6) where cytidine is replaced with guanine.

[0156] Accordingly, OCIF5 has a mutation ~~and~~ where asparagine (Asn) at amino acid number [[-19]] 3 in OCIF (~~sequence number~~ SEQ ID NO: 5) is replaced with lysine (Lys). The mutation seems to be located in the signal sequence and ~~have~~ has no essential effect on ~~secreted~~ the secretion of OCIF5.

[0157] The OCIF5 cDNA has the latter portion (about 1.8 kb) of intron 2 between nucleotide number 400 and nucleotide number 401 in OCIF cDNA (~~sequence number~~ SEQ ID NO: 6). The open reading frame stops in the latter portion of intron 2.

[0158] Accordingly, OCIF5 has an additional novel amino acid sequence containing 12 amino acids after alanine (Ala) at amino acid number 133 ~~112~~ in OCIF (~~sequence number~~ SEQ ID NO: 5).

EXAMPLE 21

[0159] Production of OCIF variants

[0160] i) Construction of the plasmid for expressing OCIF variants

[0161] ~~The~~ [[p]]Plasmids containing OCIF2 or OCIF3 cDNA ~~was~~ were obtained as described in EXAMPLE 20 and called pBKOCIF2 and pBKOCIF3, ~~respectively. pBKOCIF2~~ respectively. pBKOCIF2 and pBKOCIF3 were digested with restriction enzymes BamHI and XhoI. The OCIF2 and OCIF3 cDNA inserts were separated by agarose gel electrophoresis and purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The purified OCIF2 and

OCIF3 cDNA inserts were individually ligated using a DNA ligation kit ver. 2 (Takara Shuzo) to the expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes BamHI and ~~XhoI. E.~~ XhoI. *E. coli*[[.]] strain DH5 α (Gibco BRL) was transformed with the ligation mixture.

[0162] The plasmid containing OCIF4 cDNA was obtained as described in EXAMPLE 20 and called pBKOCIF4. pBKOCIF4 was digested with restriction enzymes SpeI and XhoI (Takara Shuzo). The OCIF4 cDNA insert was separated by agarose gel electrophoresis, and purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The purified OCIF4 cDNA insert was ligated using a DNA ligation kit ver. 2 (Takara Shuzo) to an expression vector pCEP4 (Invitrogen) that had been digested with restriction enzymes NheI and XhoI (Takara Shuzo). *E. coli* strain DH5 α (Gibco BRL) was transformed with the ligation mixture.

[0163] The plasmid containing OCIF5 cDNA was obtained as described in EXAMPLE 20 and called pBKOCIF5. pBKOCIF5 was digested with the restriction enzyme HindIII (Takara Shuzo). The ~~5'portion~~ 5' portion of the coding region in the OCIF5 cDNA insert was separated by agarose gel electrophoresis and purified from the gel using ~~a QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The OCIF expression plasmid, pCEPOCIF, obtained in EXAMPLE 13-i) was digested with the restriction enzyme HindIII (Takara Shuzo). The ~~5'portion~~ 5' portion of the coding region in the OCIF cDNA was removed. The rest of the plasmid that contains pCEP vector and the ~~3'portion~~ 3' portion of the coding region of OCIF cDNA was called pCEPOCIF-3'. pCEPOCIF3' was separated by agarose gel electrophoresis and purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The OCIF5 cDNA HindIII fragment and pCEPOCIF-3' were ligated using a DNA ligation kit ver. 2 (Takara Shuzo). *E. coli*[[.]] strain DH5 α (Gibco BRL) was transformed with the ligation mixture.

[0164] The ~~obtained~~ transformants obtained were grown at 37 °C overnight and the OCIF variant[[s]] expression plasmids (pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5) were purified using ~~QIAGEN~~ QIAGEN™ columns (QIAGEN). These OCIF-variant[[s]]-expression plasmids were precipitated with ethanol, dissolved in sterile distilled water, and used in the experiments described below.

[0165] ii) Transient expression of OCIF variant cDNAs and analysis of the biological activity of recombinant OCIF variants.

[0166] Recombinant OCIF variants were produced using the expression plasmids, pCEPOCIF2, pCEPOCIF3, pCEPOCIF4, and pCEPOCIF5 as described in EXAMPLE 21-i) according to the method described in EXAMPLE 13-ii). The biological activities of recombinant OCIF variants were analyzed. The results were that these OCIF variants (OCIF2, OCIF3, OCIF4, and OCIF5) had weak activity.

EXAMPLE 22

[0167] Preparation of OCIF mutants

[0168] i) Construction of a plasmid vector for subcloning cDNAs encoding OCIF mutants

[0169] The plasmid vector (~~5μg~~ 5 μg) described in EXAMPLE 11 was digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI (Takara Shuzo). The digested DNA was subjected to preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 1.6 kilobase pairs (kb) that contained the entire coding sequence for OCIF was purified from the gel using a ~~QIAEX~~ QIAEX™ gel extraction kit (QIAGEN). The purified DNA was dissolved in 20 ~~μl~~ μL of sterile distilled water. This solution was designated DNA solution l. ~~pBluescript II SK +~~ pBLUESCRIPT II SK +™ (3 μg) (Stratagene) was digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI (Takara Shuzo). The digested DNA was subjected to preparative agarose gel electrophoresis. A DNA fragment with an approximate size of 3.0 kb was purified from the gel using a ~~QIAEX~~ QIAEX™ DNA extraction kit (QIAGEN). The purified DNA was dissolved in 20 ~~μl~~ μL of sterile distilled water. This solution was designated DNA ~~solution 2. One~~ solution 2. One microliter of DNA solution 2, 4 ~~μl~~ μL of DNA solution 1 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 (Takara Shuzo) were mixed and incubated at 16°C for 30 min. (The ligation mixture was used in the transformation of *E. coli* in [[a]] the manner described below). Conditions for transformation of *E. coli* were as follows. One hundred microliters of competent *E. coli* strain DH5 α cells (GIBCO BRL) and ~~5μl~~ 5 μL of the ligation mixture were mixed in a sterile 15-ml tube (IWAKI glass). The tube was kept on ice for 30 min. After incubation for 45 sec at 42°C, ~~to the cells was added~~ 250 ~~μl~~ μL of L broth (1% Tryptone, 0.5% yeast extract, 1% NaCl) was added to the cells. The cell suspension was then incubated for 1 hr. at 37°C with shaking. Fifty microliters of the cell suspension was plated onto

an L-agar plate containing ~~5µg~~ 5 µg/ml of ampicillin. The plate was incubated overnight at 37°C.

[0170] Six colonies which grew on the plate were each incubated in 2 ml ~~each~~ of L-broth containing 50 µg/ml ampicillin overnight at 37°C with shaking. The structure of the plasmids in the colonies was analyzed. A plasmid in which the 1.6-kb DNA fragment containing the entire OCIF cDNA is inserted between the digestion sites of ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI of ~~pBluescript II SK +~~ pBLUESCRIPT II SK +™ was obtained and designated as pSK + -OCIF.

[0171] ii) Preparation of mutants in which one of the Cys residues in OCIF is replaced with a Ser residue

[0172] 1) Introduction of mutations into OCIF cDNA

[0173] OCIF mutants were prepared in which one of the five Cys residues present in OCIF at positions 174, 181, 256, 298 and 379 (in ~~SEQUENCE NO~~ SEQ ID NO: 4) was replaced with a Ser residue and were designated ~~OCIF-C19S(174Cys~~ OCIF-C19S (174Cys to Ser), OCIF-C20S (181Cys to Ser), OCIF-C21S (256Cys to Ser), OCIF-C22S (298Cys to Ser) and OCIF-C23S (379Cys to Ser), respectively. The amino acid sequences of these mutants are provided in the sequence listing as SEQ ID NOs: 62, 63, 64, 65, and 66, respectively.

[0174] To prepare the mutants, nucleotides encoding the corresponding Cys residues were replaced with those encoding Ser. Mutagenesis was carried out by a two-step polymerase chain reaction (PCR). The first step of the PCRs consisted of two reactions, PCR 1 and PCR 2.

PCR [[I]] 1	10x Ex Taq Buffer (Takara Shuzo)	10	~~µl~~ µL
	2.5 mM solution of dNTPs	8	~~µl~~ µL
	the plasmid vector described in EXAMPLE 11 (8ng/ml)	2	~~µl~~ µL
	sterile distilled water	73.5	~~µl~~ µL
	20 µM solution of primer 1	5	~~µl~~ µL
	100 µ M solution of primer 2 (for mutagenesis)	1	~~µl~~ µL

	Ex Taq (Takara Shuzo)	0.5	~~μl~~ μL
PCR 2	10x Ex Taq Buffer (Takara Shuzo)	10	~~μl~~ μL
	2.5 mM solution of dNTPs	8	~~μl~~ μL
	the plasmid vector described in EXAMPLE 11 (8ng/ml)	2	~~μl~~ μL
	sterile distilled water	73.5	~~μl~~ μL
	20 μM solution of primer 3	5	~~μl~~ μL
	100 μM solution of primer 4 (for mutagenesis)	1	~~μl~~ μL
	Ex Taq (Takara Shuzo)	0.5	~~μl~~ μL

[0175] Specific sets of primers were used for each mutation and other components were unchanged. Primers used for the reactions are shown in Table 10. The nucleotide sequences of the primers are shown in ~~SEQUENCE NO:~~ SEQ ID NOs: 20, 23, 27 and 30-40. The PCRs were performed under the following conditions. An initial denaturation step at 97°C for 3 min was followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 3 min. After these amplification cycles, final extension was performed at 70°C for 5 min. The sizes of the PCR products were confirmed by agarose gel electrophoresis of the reaction solutions. After the first PCR, excess primers were removed using an Amicon ~~microcon~~ MICROCON™ (Amicon). The final volume of the solutions that contained the PCR products were made to 50 ~~μl~~ μL with sterile distilled water. These purified PCR products were used for the second PCR (PCR 3).

PCR 3	10x Ex Taq Buffer (Takara Shuzo)	10	~~μl~~ μL

2.5 mM solution of DNTPS	8	~~μl~~ μL
solution containing DNA fragment obtained from PCR 1	5	~~μl~~ μL
solution containing DNA fragment obtained from PCR 2	5	~~μl~~ μL
sterile distilled water	61.5	~~μl~~ μL
20 μM solution of primer 1	5	~~μl~~ μL
20 μM solution of primer 3	5	~~μl~~ μL
Ex Taq (Takara Shuzo)	0.5	~~μl~~ μL

Table 10

mutants	primer-1	primer-2	primer-3	primer-4
~~OCIF-C19s~~ OCIF-C19S	IF 10	C19SR	IF 3	C19SF
OCIF-C20S	IF 10	C20SR	IF 3	C20SF
OCIF-C21S	IF 10	C21SR	IF 3	C21SF
OCIF-C22S	IF 10	C22SR	IF 14	C22SF
OCIF-C23S	IF 6	C23SR	IF 14	C23SF

[0176] The reaction conditions were exactly the same as those for PCR 1 or PCR 2. The sizes of the PCR [[products-was]] products were confirmed by ~~1.0 %~~ 1.0% or ~~1.5 %~~ 1.5% agarose gel electrophoresis. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 ~~μl~~ μL of sterile distilled water. The solutions containing DNA fragments with mutations C19S, C20S, C21S, C22S and C23S were designated as DNA solution A, DNA solution B, DNA solution C, DNA solution D and DNA solution E, respectively.

[0177] The DNA fragment which is contained in solution A (20 ~~μl~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Sph I~~ SphI (Takara Shuzo). A DNA fragment with an approximate size of 400 base pairs (bp) was extracted from a preparative agarose gel and

dissolved in 20 ~~μ-l~~ μL of sterile distilled water. This DNA solution was designated DNA solution ~~3. Two~~ 3. Two micrograms of pSK + -OCIF were digested with restriction enzymes ~~Nde I~~ NdeI and ~~Sph I~~ SphI. A DNA fragment with an approximate size of 4.2 kb was purified from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ-l~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution ~~4. Two~~ 4. Two microliters of DNA solution 3, 3 ~~μ-l~~ μL of DNA solution 4 and 5 ~~μ-l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5α cells were transformed with 5 ~~μ-l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C19S.

[0178] The DNA fragment contained in solution B (20 ~~μ-l~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Sph I~~ SphI. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ-l~~ μL of sterile distilled water. This DNA solution was designated DNA solution ~~5. Two~~ 5. Two microliters of DNA solution 5, 3 ~~μ-l~~ μL of DNA solution 4 and 5 ~~μ-l~~μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain ~~DHS~~ DH5 α cells were transformed with ~~μ-l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named ~~pSK-OCIF-C2OS~~ pSK-OCIF-C20S.

[0179] The DNA fragment which is contained in solution C (20 ~~μL~~μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Sph I~~ SphI. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μL~~μL of sterile distilled water. This DNA solution was designated DNA solution ~~6. Two~~ 6. Two microliters of DNA solution 6, 3 ~~μ-l~~ μL of DNA solution 4 and 5 ~~μ-l~~ μL of ligation buffer I from a ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μ-l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing

plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C21S.

[0180] The DNA fragment which is contained in solution D (20 ~~μ l~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Bst PI~~ BstPI. A DNA fragment with an approximate size of 600 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution 7. Two micrograms of pSK + -OCIF were digested with restriction enzymes ~~Nde I~~ NdeI and ~~Bst PI~~ BstPI. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution 8. Two microliters of DNA solution 7, 3 ~~μ l~~ μL of DNA solution 8 and 5 ~~μ l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μ l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA in which the 600-bp ~~Nde I-BstPI~~ NdeI-BstPI fragment with the mutation (the C22S mutation) is substituted for the 600-bp ~~Nde I-Bst PI~~ NdeI-BstPI fragment of ~~pSK+ -OCIF~~ pSK + -OCIF by analyzing the DNA structure. DNA structure was analyzed by restriction enzyme mapping and by ~~DNA/sequencing~~ DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C22S.

[0181] The DNA fragment which is contained in solution E (20 ~~μ l~~ μL) was digested with restriction enzymes ~~Bst PI~~ BstPI and ~~Eco RV~~ EcoRV. A DNA fragment with an approximate size of 120 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution ~~9. Two~~ 9. Two micrograms of pSK + -OCIF were digested with restriction enzymes ~~Bst EII~~ BstEII and ~~Eco RV~~ EcoRV. A DNA fragment with an approximate size of 4.5 kb was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution 10. Two microliters of DNA solution 9, 3 ~~μ l~~ μL of DNA solution 10 and 5 ~~μ l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μ l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA.

DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-C23S.

[0182] 2) Construction of vectors for expressing the OCIF mutants

[0183] pSK-OCIF-Cl9S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S and pSK-OCIF-C23S were digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI. The 1.6 kb ~~Bam HI-Xho I~~ BamHI-XhoI DNA fragment encoding each OCIF mutant was isolated and dissolved in 20 ~~µ l~~ µL of sterile distilled water. The DNA solutions that contain 1.6 kb cDNA fragments derived from pSK-OCIF-Cl9S, pSK-OCIF-C20S, ~~pSK-OCIF-c2lS~~ pSK-OCIF-C2lS, pSK-OCIF-C22S and pSK-OCIF-C23S were designated C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution and C23S DNA solution, respectively. Five micrograms of expression vector pCEP 4 (Invitrogen) ~~was~~ were digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI. A DNA fragment with an approximate size of 10 kb was purified and dissolved in 40 ~~µ l~~ µL of sterile distilled water. This DNA solution was designated as pCEP 4 DNA solution. One microliter of pCEP 4 DNA solution and 6 ~~µ l~~ µL of either C19S DNA solution, C20S DNA solution, C21S DNA solution, C22S DNA solution or C23S DNA solution were independently mixed with 7 ~~µ l~~ µL of ligation buffer I from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent *E. coli* strain DH5 α cells (100 ~~µL~~µL) were transformed with 7 ~~µ l~~ µL of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmid in which a 1.6-kb cDNA fragment is inserted between the recognition sites of ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI of pCEP 4 by analyzing the DNA structure. The ~~plasmide~~ plasmids which were obtained containing the cDNA encoding OCIF-C19S, OCIF-C20S, OCIF-C21S, OCIF-C22S and OCIF-C23S (SEQ ID NOs: 83, 84, 85, 86, and 87, respectively) were designated ~~pCEP4-OCIF-Cl9S~~ pCEP4-OCIF-C19S, pCEP4-OCIF-C20S, pCEP4-OCIF-C21S, pCEP4-OCIF-C22S and ~~CEP4-OCIF-C23S~~ pCEP4-OCIF-C23S, ~~respectively,~~ respectively.

[0184] ~~[[ii]]~~ iii) Preparation of domain-deletion mutants of OCIF

[0185] (1) deletion mutagenesis of OCIF cDNA

[0186] A series of OCIF mutants with deletions from Thr 2 to Ala 42, from Pro 43 to Cys 84, from Glu 85 to Lys 122, from Arg 123 to Cys 164, from Asp 177 to Gln 251 or from Ile 252 to His 326 were prepared (positions of the amino acid residues are shown in ~~SEQUENCE NO:~~ SEQ ID NO: 4). These mutants were designated as OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2, respectively, and assigned SEQ ID NOs: 67, 68, 69, 70, 71, and 72, respectively.

[0187] Mutagenesis was performed by two-step PCR as described in EXAMPLE 22-[[(]]ii). The primer sets for the reactions are shown in Table 11 and the nucleotide sequences of the primers are shown in ~~SEQUENCE NO:~~ SEQ ID NOs: 19, 25, 40-53 and 54.

Table 11

mutants	primer-1	primer-2	primer-3	primer-4
~~OCIF-DCRI~~ OCIF-DCR1	~~XhoI~~ XhoI F	~~DCRIR~~ DCR1R	IF 2	DCR1F
OCIF-DCR2	~~XhoI~~ XhoI F	DCR2R	IF 2	DCR2F
OCIF-DCR3	~~XhoI~~ XhoI F	DCR3R	IF 2	DCR3F
OCIF-DCR4	~~XhoI~~ XhoI F	DCR4R	IF 16	DCR4F
~~OCIF-DDDI~~ OCIF-DDD1	IF 8	~~DDDLR~~ DDD1R	IF 14	DDD1F
OCIF-DDD2	IF 8	DDD2R	IF 14	DDD2F

[0188] The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40 ~~μ l~~ μL of sterile distilled water. Solutions of DNA fragments coding for portions of OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 were designated DNA solutions F, G, H, I, J and K, respectively.

[0189] The DNA fragment ~~which is~~ contained in solution F (20 ~~μ l~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Xho I~~ XhoI. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution 11. Two micrograms of ~~pSK+ -OCIF~~ pSK + -OCIF ~~was~~ were digested

with restriction enzymes ~~Nde I~~ NdeI and ~~Xho I~~ XhoI. A DNA fragment with an approximate size of 4.0 kb was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μl~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 12. Two microliters of DNA solution 11, 3 ~~μl~~ μL of DNA solution 12 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μl~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR1.

[0190] The DNA fragment which is contained in solution G (20 ~~μl~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Xho I~~ XhoI. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in ~~20μl~~ 20 μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 13. Two microliters of DNA solution 13, 3 ~~μl~~ μL of DNA solution 12 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and ~~the~~ ligation was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μl~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR2.

[0191] The DNA fragment ~~which is~~ contained in solution H (20 ~~μl~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~Xho I. A~~ XhoI. A DNA fragment with an approximate size of 500 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μl~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 14. Two microliters of DNA solution 14, 3 ~~μl~~ μL of DNA solution 12 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μl~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing a plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR3.

[0192] The DNA fragment ~~which is~~ contained in solution I (20 ~~μl~~ μL) was digested with restriction enzymes ~~Xho I~~ XhoI and ~~Sph I. A~~ SphI. A DNA fragment with an approximate size of 900 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μL~~μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 15. Two micrograms of ~~pSK+-OCIF~~ pSK + -OCIF ~~was~~ were digested with restriction enzymes ~~Xho I~~ XhoI and ~~Sph I. A~~ SphI. A DNA fragment with an approximate size of 3.6 kb was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μl~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 16. Two microliters of DNA solution 15, 3 ~~μl~~ μL of DNA solution 16 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μl~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing ~~a~~ plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DCR4.

[0193] The DNA fragment ~~which is~~ contained in solution J (20 ~~μl~~ μL) was digested with restriction enzymes ~~BstP I~~ BstPI and ~~Nde I~~ NdeI. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μL~~μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 17. Two microliters of DNA solution 17, 3 ~~μL~~μL of DNA solution 8 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μL~~μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing ~~a~~ plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD1.

[0194] The DNA fragment ~~which is~~ contained in solution K (20 ~~μl~~ μL) was digested with restriction enzymes ~~Nde I~~ NdeI and ~~BstP I. A~~ BstPI. A DNA fragment with an approximate size of 400 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μl~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ DNA solution 18. Two microliters of DNA solution 18, 3 ~~μl~~ μL of DNA solution 8 and 5 ~~μl~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and the ligation

reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μ-l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing ~~a~~ plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named pSK-OCIF-DDD2.

[0195] 2) Construction of vectors for expressing the OCIF mutants

[0196] ~~pSK-OCIF-DCRI~~ pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, ~~PSK-OCIF-DDDI~~ pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I. The Bam HI-Xbo I~~ XhoI. The BamHI-XhoI DNA fragment containing the entire coding sequence for each OCIF mutant was isolated and dissolved in 20 ~~μ-l~~ μL of sterile distilled water. These DNA solutions that contain the ~~Bam HI-Xho I~~ BamHI-XhoI fragment derived from ~~pSK-OCIF-DCRI~~ pSK-OCIF-DCR1, pSK-OCIF[[,]]-DCR2, pSK-OCIF-DCR3, pSK-OCIF-DCR4, ~~PSK-OCIF-DDDI~~ pSK-OCIF-DDD1 and pSK-OCIF-DDD2 were designated DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, DDD1 DNA solution and DDD2 DNA solution, respectively. One microliter of pCEP 4 DNA solution and 6 ~~μ-l~~ μL of either ~~DCRI~~ DCR1 DNA solution, DCR2 DNA solution, DCR3 DNA solution, DCR4 DNA solution, ~~DDDI~~ DDD1 DNA solution or DDD2 DNA solution were independently mixed with 7 ~~μ-l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent *E. coli* strain DH5 α cells (100 ~~μ-l~~ μL) were transformed with 7 ~~μ-l~~ μL of each ligation mixture. Ampicillin-resistant transformants were screened for a clone containing ~~a~~ plasmid DNA in which the DNA fragment with deletions is inserted between the recognition sites of ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-DCR1, OCIF-DCR2, OCIF-DCR3, OCIF-DCR4, OCIF-DDD1 and OCIF-DDD2 (SEQ ID NOs: 88, 89, 90, 91, 92, and 93, respectively) were designated pCEP4-OCIF-DCR1, pCEP4-OCIF-DCR2, pCEP4-OCIF-DCR3, pCEP4-OCIF-DCR4, pCEP4-OCIF-DDD1 and pCEP4-OCIF-DDD2, respectively.

[0197] [[iii]] iv) Preparation of OCIF with C-terminal domain truncation

[0198] [[(1)]] (1) mutagenesis of OCIF cDNA

[0199] A series of OCIF mutants with deletions from Cys at amino acid residue 379 to Leu 380, from Ser 331 to Leu 380, from Asp 252 to Leu 380, from Asp 177 to Leu 380, from Arg 123 to Leu 380 and from Cys 86 to Leu 380 was prepared. Positions of the amino acid residues are shown in ~~SEQUENCE NO:~~ SEQ ID NO: ~~4. These~~ 4. These mutants were designated as OCIF-CL, OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3, respectively, and assigned SEQ ID NOs: 73, 74, 75, 76, 77, and 78, respectively.

[0200] Mutagenesis for OCIF-CL was performed by the two-step ~~PGR~~ PCR as described in EXAMPLE 22-[[(]]ii). The primer set for the reaction is shown in Table 12. The nucleotide sequences of the primers are shown in SEQ ID NOs: 23, 40, 55, and ~~66. The~~ 66. The final PCR products were precipitated with ethanol, dried under vacuum and dissolved in 40 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated solution L.

[0201] The DNA fragment ~~which is~~ contained in solution L (20 ~~μ l~~ μL) was digested with restriction enzymes ~~BstP I~~ BstPI and ~~Eco RV~~ EcoRV. A DNA fragment with an approximate size of 100 bp was extracted from a preparative agarose gel ~~with~~ using a ~~QIAEX~~ QIAEX™ gel extraction kit and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated DNA solution 19. Two microliters of DNA solution 19, 3 ~~μ l~~ μL of DNA solution 10 (described in EXAMPLE 22-[[(]]ii)) and 5 ~~μ l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were mixed and ligation reaction was carried out. Competent *E. coli* strain DH5 α cells were transformed with 5 ~~μ l~~ μL of the ligation mixture. Ampicillin-resistant transformants were screened for a clone containing [[a]] plasmid DNA. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmid thus obtained was named ~~pSK-OCIF-CL. Mutagenesis~~ pSK-OCIF-CL. Mutagenesis of OCIF cDNA to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 was performed by a one-step PCR reaction.

[0202] PCR reactions for mutagenesis to prepare OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4 and OCIF-CCR3 were as follows:

10x Ex Taq Buffer (Takara Shuzo)	10 ~~μ l~~ μL
2.5 mM solution of dNTPs	8 ~~μ l~~ μL
the plasmid vector containing the entire OCIF cDNA described in EXAMPLE 11	2 ~~μ l~~ μL

(~~8ng~~ 8 ng/ml)

sterile distilled water	73.5 ~~μ l~~ μL
20 ~~μ M~~ μM solution of primer OCIF Xho F	5 ~~μ l~~ μL
100 μM solution of primer (for mutagenesis)	1 ~~μl~~μL
Ex Taq (Takara Shuzo)	0.5 ~~μl~~μL

Table 12

mutants	primer-1	primer-2	primer-3	primer-4
OCIF-CL	IF 6	CL R	IF 14	CL F

[0203] Specific primers were used for each mutagenesis and other components were unchanged.

[0204] Primers used for the mutagenesis are shown in Table 13. Their nucleotide sequences are shown in ~~SEQUENCE NO:~~ SEQ ID NOs: 57-61. The components of each PCR were mixed in a microcentrifuge tube and PCR was performed as follows. The microcentrifuge tubes were treated for 3 minutes at 97°C and then incubated sequentially, for 30 seconds at 95°C, 30 seconds at 50°C and 3 minutes at 70°C. This three-step incubation procedure was repeated 25 times, and after that, the tubes were incubated for 5 minutes at 70°C. An aliquot of the reaction mixture was removed from each tube and analyzed by ~~an~~ agarose gel electrophoresis to confirm the size of each product.

[0205] Excess primers in the PCRs were removed using an Amicon ~~microcon~~ MICROCON™ (Amicon) after completion of the reaction. The DNA fragments were precipitated with ethanol, dried under vacuum and dissolved in 40 ~~μ l~~ μL of sterile distilled water. The DNA fragment in each DNA solution was digested with ~~restrictive~~ restriction enzymes ~~Xho I~~ XhoI and ~~Bam HI~~ BamHI. After the reactions, DNA was precipitated with ethanol, dried under vacuum and dissolved in 20 ~~μ l~~ μL of sterile distilled water.

[0206] The ~~Solutions~~ solutions containing the DNA fragment with the CC deletion, the CDD2 deletion, the CDD1 deletion, the CCR4 deletion and the CCR3 deletion were designated CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution and CCR3 DNA solution, respectively.

Table 13

mutants	primers for the mutagenesis
OCIF-CC	CC R
OCIF-CDD2	CDD2 R
OCIF-CDD1	CDD1 R
OCIF-CCR4	CCR4 R
OCIF-CCR3	CCR3 R

[0207] (2) Construction of vectors for expressing the OCIF mutants

[0208] pSK-OCIF-CL was digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI. The ~~Bam HI-Xho I~~ BamHI-XhoI DNA fragment containing the entire coding sequence for OCIF-CL was isolated and dissolved in 20 ~~μ l~~ μL of sterile distilled water. This DNA solution was designated ~~as~~ CL DNA solution. One microliter of pCEP 4 DNA solution and 6 ~~μ l~~ μL of either CL DNA solution, CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution or CCR3 DNA solution were independently mixed with 7 ~~μ l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent *E. coli* strain DH5α cells (100 ~~μ l~~ μL) were transformed with 7 ~~μ l~~ μL of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids which have the desirable mutations in the OCIF cDNA by analyzing the DNA structure. In each plasmid, the OCIF cDNA fragment having a deletion ~~were~~ was inserted between the recognition sites of ~~Xho I~~ XhoI and ~~Bam HI~~ BamHI of pCEP 4. The plasmids containing the cDNA encoding OCIF-CL, OCIF-CC, OCIF-CDD1, OCIF-CDD2, OCIF-CCR4 and OCIF-CCR3 (SEQ ID NOs: 94, 95, 96, 97, 98, and 99, respectively) were designated pCEP4-OCIF-CL, pCEP4-OCIF-CC, pCEP4-OCIF-CDD2, pCEP4-OCIF-CDD1, pCEP4-OCIF-CCR4 and pCEP4-OCIF-CCR3, respectively.

[0209] v) Preparation of OCIF mutants with C-terminal truncations

[0210] (1) Introduction of C-terminal truncations to OCIF

[0211] A series of OCIF mutants with C-terminal truncations was prepared. An OCIF mutant in which 10 residues ~~of~~ from Gln at 371 to Leu at 380 ~~are~~ were replaced with 2 residues ~~of~~ (Leu-Val) was designated OCIF-CBst (SEQ ID NO: 79). An OCIF mutant in which 83 residues from Cys 298 to Leu 380 ~~are~~ were replaced with 3 residues ~~of~~ (Ser-Leu-Asp) was designated OCIF-CSph (SEQ ID NO: 80). An OCIF mutant in which 214 residues ~~of~~ from Asn 167 to Leu 380 ~~are~~ were removed was designated OCIF-CBsp (SEQ ID NO: 81). An OCIF mutant in which 319 residues ~~of~~ from Asp 62 to Leu 380 ~~are~~ were replaced with 2 residues ~~of~~ (Leu-Val) was designated OCIF-CPst (SEQ ID NO: 82). Positions of the amino acid residues are shown in ~~SEQUENCE NO:~~ SEQ ID NO: 4.

[0212] Two micrograms each of pSK + -OCIF ~~was~~ were digested with ~~Bst PI~~ BstPI, ~~Sph I~~ SphI, PstI (Takara Shuzo) or ~~BspEI~~ BspEI (New England Biolabs) followed by phenol extraction and ethanol precipitation. The precipitated DNA was dissolved in 10 ~~μ l~~ μL of sterile distilled water. The [[E]]ends of the DNAs in 2 ~~μ l~~ μL of each solution were blunted using a DNA blunting kit in a final volume[[s]] of 5 ~~μ l~~ μL. To the reaction mixtures, 1 ~~μ g~~ μg (1 ~~μ l~~ μL) of an Amber codon-containing ~~Xba I~~ XbaI linker (5'-CTAGTCTAGACTAG-3') and 6 ~~μ l~~ μL of ligation buffer I from a DNA ligation kit ver. 2 were added.

[0213] After the ligation reactions, 6 ~~μ l~~ μL each of the reaction mixtures was used to transform *E. coli* strain DH5 α. Ampicillin-resistant transformants were screened for clones containing plasmids. DNA structure was analyzed by restriction enzyme mapping and by DNA sequencing. The plasmids thus obtained were named pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst, respectively.

[0214] (2) Construction of vectors expressing the OCIF mutants

[0215] pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp and pSK-OCIF-CPst were digested with restriction enzymes ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI. The 1.5 kb DNA fragment containing the entire coding sequence for each OCIF mutant was isolated and dissolved in 20 ~~μ l~~ μL of sterile distilled water. These DNA solutions that contained the ~~Bam HI-XhoI~~ BamHI-XhoI fragment derived from pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp or pSK-

OCIF-CPst were designated ~~as~~ CBst DNA solution, CSph DNA solution, CBsp DNA solution and CPst DNA solution, respectively. One microliter of pCEP 4 DNA solution (described in EXAMPLE 22-ii)) and 6 ~~μ 1~~ μL of either CBst DNA solution, CSph DNA solution, CBsp DNA solution or CPst DNA solution were independently mixed with 7 ~~μ 1~~ μL of ligation buffer I ~~of~~ from a DNA ligation kit ver. 2 and the ligation reactions were carried out. Competent *E. coli* strain DH5 α cells (100 ~~μ 1~~ μL) were transformed with 7 ~~μ 1~~ μL of each ligation mixture. Ampicillin-resistant transformants were screened for clones containing plasmids in which the cDNA fragment ~~is~~ was inserted between the recognition sites of ~~Bam HI~~ BamHI and ~~Xho I~~ XhoI of pCEP 4 by analyzing the DNA structure. The plasmids containing the cDNA encoding OCIF-CBst, OCIF-CSph, OCIF-CBsp or OCIF-CPst (SEQ ID NOs: 100, 101, 102, and 103, respectively) were designated pCEP4-OCIF-CBst, ~~pCEP4-OCIF- CSph~~ pCEP4-OCIF-CSph, pCEP4-OCIF-CBsp and pCEP4-OCIF-CPst, respectively.

[0216] [[v]] vi) Preparation of vectors for expressing the OCIF mutants

[0217] *E. coli* clones harboring the expression vectors for OCIF mutants (a total of 21 clones) were grown and the vectors were purified by using ~~QIAGEN~~ QIAGEN™ columns (QIAGEN). All the expression vectors were precipitated with ethanol and dissolved in appropriate volumes of sterile distilled water and used for further ~~manipupations~~ manipulations shown below.

[0218] [[vi]] vii) Transient expression of the cDNAs for OCIF mutants and biological activities of the mutants

[0219] OCIF mutants were produced using the expression vectors prepared in EXAMPLE ~~22-v)~~ 22-vi). The method was essentially the same as described in EXAMPLE 13. Only the modified points are described below. ~~A 24-well plate was used for the DNA transfection.~~ ~~$2x10^5$~~ 2×10^5 cells of 293/EBNA suspended in IMDM containing 10% fetal bovine serum were seeded into each well of ~~the~~ a 24 well plate. One microgram of purified vector DNA and 4 ~~μ 1~~ μL of lipofectamine were used for each transfection. A [[M]]mixture of ~~an~~ the expression vector and lipofectamine in ~~OPTI-MEM~~ OPTI-MEM™ (GIBCO BRL) in a final volume of 0.5 ml was added to the cells in a well. After the cells were incubated at 37°C for 24 hr in ~~a~~ 5% CO_2 ~~incubator~~, the medium was replaced with 0.5 ml of ~~Ex-cell~~ EXCELL™ 301 medium (JSR). The cells were incubated at 37°C for a further 48 ~~more~~ hours in 5% CO_2. The conditioned medium was collected and used ~~for~~ in assays for in vitro biological activity. The nucleotide sequences of

cDNAs for the OCIF mutants are shown in ~~SEQUENCE NO:~~ SEQ ID NOs: 83-103. The deduced amino acid sequences for the OCIF mutants are shown in ~~SEQUENCE NO:~~ SEQ ID NOs: 62-82. The assay for in vitro biological activity was performed as described in EXAMPLE 13. The [[A]]antigen concentration of each conditioned medium was determined by ELISA as described in EXAMPLE 24. Table 14 shows ~~specific~~ the activity of each mutant relative to that of the unaltered OCIF.

Table 14

mutants	activity
the unaltered ~~OIF~~ OCIF	+ +
OCIF-C19S	+
OCIF-C20S	±
OCIF-C21S	±
OCIF-C22S	+
OCIF-C23S	+ +
OCIF-DCR1	±
OCIF-DCR2	±
OCIF-DCR3	±
OCIF-DCR4	±
~~OCIF-DDDI~~ OCIF-DDD1	+
OCIF-DDD2	±
OCIF-CL	+ +
OCIF-CC	+ +
OCIF-CDD2	+ +
OCIF-CDD1	+
OCIF-CCR4	±

OCIF-CCR3	±
OCIF-CBst	++
OCIF-CSph	++
OCIF-CBsp	±
OCIF-CPst	±

++ indicates relative activity more than 50% of that of the unaltered OCIF;

+ indicates relative activity between 10% and 50%;

± indicates relative activity less than 10%, or production level too low to determine the accurate biological activity.

[0220] viii) ~~western~~ Western blot analysis

[0221] Ten microliters of the final conditioned medium was used for western blot analysis. Ten microliters of [[the]] each sample were mixed with 10 ~~μ l~~ μL of SDS-PAGE sample buffer (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20 ~~μ g~~ μg/ml bromophenol blue, pH ~~6. 8~~ 6.8), boiled for 3 min. and subjected to 10% SDS polyacrylamide gel electrophoresis under non-reducing conditions. After the electrophoresis, the separated proteins were blotted to PVDF membrane (~~ProBlott~~® PROBLOTT™, Perkin Elmer) using a semi-dry electroblotter (BIO-RAD). The membrane was incubated at 37°C with horseradish peroxidase-labeled anti-OCIF antibodies for 2 hr. After the membrane was washed, protein bands which react with the labeled antibodies were detected using an ECL™ system (Amersham). Two protein bands with approximate molecular ~~masses~~ weights of ~~60kD~~ 60 kD and ~~120kD~~ 120 kD were detected for the unaltered OCIF. On the other hand, almost exclusively a 60 kD protein band was detected for the OCIF-C23S, OCIF-CL and ~~OCIF-CC~~ OCIF-CC mutants. Protein bands with ~~an~~ approximate ~~masses~~ weights of ~~40kD-50kD~~ 40 kD-50 kD and ~~30kD-40kD~~ 30 kD-40 kD were the major ones for OCIF-CDD2 and OCIF-CDD1, respectively. These results indicate that Cys at 379 is responsible for the dimer formation, both the monomers and the dimers maintain the biological activity and a deletion of residues from Asp at 177 to Leu at 380 does not abolish the biological activity of OCIF (positions of the amino acid ~~resare~~ residues are shown in ~~SEQUENCE NO:~~ SEQ ID NO: 4).

EXAMPLE 23

[0222] Isolation of human genomic OCIF gene

[0223] i) Screening of a human genomic library

[0224] An amplified human placenta genomic library in LAMBDA FIX™ II vector ~~purchased from~~ (Stratagene) was screened for the gene encoding human OCIF using the human OCIF cDNA as a probe. Essentially, screening was done according to the instruction manual supplied with the genomic library. The basic protocols described in *Molecular Cloning: A Laboratory Manual* ~~also were~~ were also employed to manipulate phage, *E. coli*, and DNA.

[0225] The library was titered, and ~~$1x10^6$~~ 1×10^6 pfu of phage was mixed with XL1-Blue MRA host *E. coli* cells and plated onto 20 plates (9 cm x 13 cm) with 9 ml per plate of top agarose. The plates were incubated overnight at 37°C. Filter plaque lifts were prepared using ~~Hybond-N~~ HYBOND™-N nylon membranes (Amersham). The membranes were processed by denaturation in a solution containing 1.5 M NaCl and 0.5 M NaOH for 1 minute at room temperature. The membranes were then neutralized by placing ~~successively for one minute~~ each one in 1 M Tris-HC1 (~~pH7.5~~ pH 7.5) and a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) successively for one minute each. The membranes were then transferred onto a filter paper wetted with ~~2xSSC~~ 2 x SSC. Phage DNA was fixed onto the membranes with 1200 ~~μ~~microJoules of UV energy using a STRATALINKER UV ~~crosslinker~~ CROSSLINKER™ 2400 (STRATAGENE) and the membranes were air dried. The membranes were immersed in Rapid Hybridization buffer (Amersham) and incubated for one hour at 65°C before hybridization with ^{32}P-labeled cDNA probe in the same buffer overnight at 65°C. Screening probe was prepared by labeling the OCIF cDNA with ^{32}P using the Megaprime DNA labeling system (Amersham). Approximately, ~~$5x10^5$~~ 5×10^5 cpm probe was used for each ml of hybridization buffer. After the hybridization, the membranes were rinsed in ~~2xSSC~~ 2 x SSC for five minutes at room temperature. The membranes were then washed four times, 20 minutes each time, in ~~0.5xSSC~~ 0.5 x SSC containing 0.1% SDS at 65°C. After the final wash, the membranes were dried and subjected to autoradiography at -80°C with SUPER HR-H™ X-ray film (FUJI PHOTO FILM Co., Ltd.) and an intensifying screen.

[0226] Upon examination of the autoradiograms, six positive signals were detected. Agar plugs were picked from the regions ~~corresponded~~ corresponding to these signals for phage purification. Each agar plug was soaked overnight in 0.5 ml of SM buffer containing 1% chloroform to extract phage. Each extract containing phage was diluted 1000 fold with SM buffer and an aliquot of 1 ~~μl~~ μL or 20 ~~μl~~ μL was mixed with host *E. coli* described above. The mixture was plated onto agar plates with top agarose as described above. The plates were incubated overnight at 37°C, and filter lifts were prepared, prehybridized, hybridized, washed and autoradiographed as described above. This process of phage purification was applied to all six positive signals initially detected on the autoradiograms and was repeated until all phage plaques on agar plates hybridize with the cDNA probe. After purification, agar plugs of each phage isolate were soaked in SM buffer containing 1% chloroform and stored at [[4 C]] 4°C. Six individual phage isolates were designated λ0IF3, λ0IF8, λ0IF9, λ0IF11, λ0IF12 and λ0IF17, respectively.

[0227] ii) Analysis of the genomic clones by restriction enzyme digestion and Southern blot hybridization

[0228] DNA was prepared from each phage isolate by the plate lysate method as described in *Molecular Cloning: A Laboratory Manual*. DNA prepared from each phage was digested with restriction enzymes and the fragments derived from the digestion were separated on agarose gels. The fragments were then transferred to nylon membranes and subjected to Southern blot hybridization using OCIF cDNA as a probe. The results of the analysis revealed that the six phage isolates are individual clones. Among these fragments derived from restriction enzyme digestion, those fragments which hybridized with the OCIF cDNA probe were subcloned into plasmid vectors and subjected to ~~the~~ nucleotide sequence analysis as described below.

[0229] iii) Subcloning restriction fragments derived from genomic clones into plasmid vectors and ~~determination of~~ determining their nucleotide sequence.

[0230] λ0IF8 DNA was digested with restriction enzymes EcoRI and NotI and the DNA fragments derived ~~these from~~ therefrom were separated on a 0.7% agarose gel. The 5.8 kilobase pair (kb) EcoRI/NotI fragment was extracted from the gel using a ~~QIAEX~~ QIAEX™ II Gel Extraction Kit (QIAGEN) according to the procedure recommended by the manufacturer. The 5.8 kb EcoRI/NotI fragment was ligated with ~~pBluescript II SK+~~ pBLUESCRIPT II SK+™

vector (STRATAGENE), which had been linearized with restriction enzymes EcoRI and NotI, using ~~Ready To Go~~ READY-TO-GO™ T4 DNA Ligase (Pharmacia) according to the procedure recommended by the manufacturer. Competent DH5 α *E. coli* cells (Amersham) were transformed with the recombinant plasmid and transformants were selected on L-plates containing 50 μg/ml of ampicillin.

[0231] A clone harboring the recombinant plasmid containing the ~~5. 8~~ 5.8 kb EcoRI/NotI fragment was isolated and this plasmid was termed ~~pBSG8-5.8. pBSG8-5.8~~ pBSG8-5.8. pBSG8-5.8 was digested with HindIII and a 0.9 kb DNA fragment derived from this digestion was isolated in the same manner as described above. This 0.9 kb fragment was then cloned into ~~pBluescript II SK-~~ pBLUESCRIPT II SK-™ at the HindIII site as described above. This recombinant plasmid containing 0.9 kb HindIII fragment was denoted ~~pBS8HO.9~~ pBS8H0.9.

[0232] ~~λ01F11~~ λ0IF11 DNA was digested with EcoRI and 6 kb, 3.6 kb, 2.6 kb EcoRI fragments were isolated in the same manner as described above and cloned into a ~~pBluescript II SK+~~ pBLUESCRIPT II SK+™ vector at the EcoRI site as described above. These recombinant plasmids were termed pBSG11-6, pBSG11-3.6, and pBSG11-2.6, ~~respectively. pBSG11-6~~ respectively. pBSG11-6 was digested with HindIII and the digest was ~~applied~~ separated on a ~~0.7 %~~ 0.7% agarose gel. Three fragments, 2.2 kb, 1.1 kb, and 1.05 kb in length, were extracted from the gel and cloned independently into ~~pBluescript II SK-~~ pBLUESCRIPT II SK-™ vector at the HindIII site in the same manner as described above. These recombinant plasmids were termed pBS6H2.2, pBS6H1.1 and pBS6H1.05, respectively.

[0233] The nucleotide sequence of the cloned genomic DNA was determined using a ABI ~~Dyedeoxy Terminator Cycle Sequencing Ready Reaction~~ DYEDEOXY TERMINATOR CYCLE SEQUENCING READY REACTION™ Kit (PERKIN ELMER) and a 373A DNA Sequencing system (Applied Biosystems). Plasmids pBSG8-5.8, pBS8H0.9, pBSG11-6, ~~pBSG11-3. 6, pBSG11-2. 6, pBS6H2. 2~~ pBSG11-3.6, pBSG11-2.6, pBS6H2.2, pBS6H1.1 and pBS6H1.05 were prepared according to the alkaline-SDS procedure as described in *Molecular Cloning: A Laboratory Manual* and used as templates for ~~the~~ DNA sequence analysis. The [[N]]nucleotide sequence of the human OCIF gene ~~was~~ is presented in ~~Sequence No~~ SEQ ID NO: 104 and ~~Sequence No~~ SEQ ID NO: 105. The nucleotide sequence of the DNA, between exon 1 and exon [[2]] 2, was not entirely determined. There is a stretch of approximately 17 kb

~~of nucleotides~~ between the sequences given in ~~sequence No.~~ SEQ ID NO: 104 and ~~sequence No.~~ SEQ ID NO: 105.

EXAMPLE 24

[0234] Quantitation of OCIF by EIA

[0235] i) Preparation of anti-OCIF antibody

[0236] Male ~~JW~~ Japanese white rabbits (Kitayama ~~LABES~~ Labs Co., LTD) weighing 2.5-3.0 kg were used in immunization for preparing antisera. ~~Three male JW rabbits (Kitayama LABES Co., LTD) weighing 2.5-3.0 kg were used for immunization.~~ For immunization, an emulsion was prepared by mixing an equal volume of rOCIF (200 μg/ml) and complete Freund's adjuvant (Difco, Cat. 0638-60-7). ~~The~~ Three rabbits were immunized subcutaneously six times at ~~the interval of~~ one week intervals with 1 ml of emulsion per injection. ~~The rabbits were injected six times at the Interval of seven days subcutaneously.~~ Whole blood was obtained ten days after the final immunization and serum was ~~separated~~ isolated. Antibody was purified from serum as follows. Antiserum was diluted two-fold with PBS. After adding ammonium sulfate at a final concentration of 40% w/v, the antiserum was allowed to stand at 4°C for 1 hr. The [[P]]precipitate obtained by centrifugation at 8000 x g for 20 min. was dissolved in a small volume of PBS and was dialyzed against PBS. The resultant ~~resulting~~ solution was loaded onto a Protein ~~G-Sepharose~~ G-SEPHAROSE™ column (Pharmacia). After washing with PBS, absorbed immunoglobulin G was eluted with 0.1 M ~~glycine-HCL~~ glycine-HCl buffer (pH 3.0). The [[E]]eluate[[s]] ~~were~~ was immediately neutralized ~~immediately~~ with 1.5 M ~~Tris-HCL~~ Tris-HCl buffer (pH [[8. 7]] 8.7) and ~~were~~ dialyzed against PBS. Protein concentration was determined by absorbance ~~At 280nm~~ at 280 nm ($E^{1\%}$ 13.5).

[0237] Horseradish peroxidase-labeled antibody was prepared using an ~~ImmunoPure~~ IMMUNOPURE™ Maleimide Activated Horseradish Peroxidase Kit (Pierce, ~~Cat.31494). Briefly~~ Cat. 31494). Briefly, one mg of IgG was incubated with 80 ~~μg~~ μl of N-succinimidyl-S-acetylthioacetate for 30 min. After deacetylation with 5 mg of hydroxylamine HC1, modified IgG was separated ~~by~~ using a polyacrylamide desalting column. The [[P]]protein pool was

mixed with one mg of maleimide-activated horseradish peroxidase ~~was~~ and incubated at room temperature for 1 hr.

[0238] ii) Quantitation of OCIF by sandwich EIA

[0239] Microtiter plates (Nunc MaxiSorp Immunoplate) were coated with rabbit anti-OCIF IgG by incubating 0.2 ~~ug~~ μg in 100 ~~ul~~ μL of 50 mM sodium bicarbonate buffer pH 9.6 at ~~4C~~ 4°C overnight. After blocking the plates by incubating for 1 hour at 37°C with 300 ~~ul~~ μL of 25% ~~BlockAce~~ BLOCKACE™/PBS (Snow Brand Milk Products[[ ]]), 100 ~~ul~~ μL samples were incubated for 2 hours at room temperature. After washing the plates three times with PBST (PBS containing 0.05% ~~Tween~~ TWEEN™ 20), 100 ~~ul~~ μL of 1:10000 diluted horseradish peroxidase-labeled anti-OCIF IgG was added and incubated for 2 hours at room temperature. The amount of OCIF was determined by incubation with 100 ~~ul~~ μL of a substrate solution (TMB, ScyTek Lab., Cat. TM4999) and measurement of the absorbance at 450 nm using an ~~ImmunoReader~~ IMMUNOREADER™ (Nunc NJ2000). Purified recombinant OCIF was used as a standard protein and a typical standard curve ~~was~~ is shown in Fig. 13.

EXAMPLE 25

[0240] Anti-OCIF monoclonal antibody

[0241] i) Preparation of a hybridoma producing anti-OCIF monoclonal antibody.

[0242] OCIF was purified to homogeneity from the culture medium of human fibroblasts, IMR-90 cells by the purification method described in ~~Example~~ EXAMPLE 11. Purified OCIF was dissolved in PBS at a concentration of 10 ~~μ g/100 μ l~~ μg/100 μL. BALB/c mice were immunized by administering this solution intraperitoneally three times every two weeks. In the first and the second immunizations, the emulsion was composed of an equal volume of OCIF and Freund's complete adjuvant ~~was administered~~. Three days after the final immunization, the spleen was ~~taken out~~ removed and lymphocytes ~~were~~ isolated and fused with mouse myeloma ~~p3x63-Ag8. 653~~ p3x63-Ag8.653 cells according to ~~the~~ conventional methods using polyethyleneglycol. Then the fused cells were cultured in HAT medium to select hybridomas. ~~Subsequently, to check whether the selected hybridomas produce anti-OCIF antibody,~~ The presence of anti-OCIF antibody in ~~each~~ the culture medium of each hybridoma[[s]] was

determined by solid phase ELISA. Briefly, ~~which was prepared by coating~~ each well ~~in~~ of a 96-well immunoplate[[s]] (Nunc) was coated with 100 ~~μ-l~~ μL of purified OCIF (10 μg/ml in 0.1 M $NaHCO_3$) and ~~by blocking each well~~ blocked with 50% ~~BlockAce~~ BLOCKACE™ (Snow Brand Milk Products Co. ~~Ltd.). The~~ Ltd.). The hybridoma clones secreting anti-OCIF antibody were established by ~~cloning 3-5 times by~~ limit dilution cloning 3-5 times and by ~~screening using the above~~ solid phase ELISA ~~screening~~. [[Among thus obtained hybridoma clones,]] Several hybridoma clones ~~with high production~~ producing high levels of anti-OCIF antibody were selected.

[0243] ii) Production of anti-OCIF monoclonal antibodies.

[0244] Each hybridoma clone secreting anti-OCIF antibody[[, which was]] obtained in EXAMPLE 25-i) was transplanted intraperitoneally into mice given Pristane (Aldrich) at a cell density of ~~1x10^6~~ 1×10^6 cells/mouse. The accumulated ascites was collected 10-14 days after ~~the~~ transplantation, thereby obtaining ~~and the ascites containing~~ anti-OCIF specific monoclonal antibody of the present invention ~~was obtained~~. Purified antibodies were obtained by Affigel protein A ~~Sepharose~~ SEPHAROSE™ chromatography (BioRad) according to the manufacturer's manual. ~~That is~~Briefly, the ascites fluid was diluted with an equal volume of a binding buffer (BioRad) and applied to a protein A column. The column was washed with a sufficient volume of binding buffer and eluted with an elution buffer (BioRad). After neutralizing, the ~~obtained~~ eluate obtained was dialyzed in water and subsequently lyophilized. The purity of the ~~obtained~~ antibody thereby obtained was analyzed by SDS/PAGE and a homogenous band with a molecular weight of about 150,000 was detected.

[0245] iii) Selection of monoclonal antibod[[y]]ies having high affinity for OCIF

[0246] Each antibody obtained in EXAMPLE 25-ii) was dissolved in PBS and the protein concentration was determined by the method of Lowry. Each antibody solution ~~with the same concentration was prepared and then serially~~ was diluted to the same concentration and then serially diluted with PBS. Monoclonal antibodies, which can recognize OCIF even at highly dilute[[d solution]] concentrations, were selected by solid phase ELISA described in EXAMPLE 25-ii). Thus, three monoclonal antibodies A1G5, E3H8 and D2F4 were ~~can be~~ selected.

[0247] iv) Determination of class and subclass of antibodies

[0248] The class and subclass of the antibodies of the present invention obtained in EXAMPLE 25-iii) were analyzed using an immunoglobulin class and subclass analysis kit (Amersham). The procedure was carried out according to ~~the protocol disclosed in~~ the kit directions. The results are shown in Table 15. The antibodies of the present invention, E3H8, A1G5 and D2F4 belong to the IgG_1, IgG_{2a} and IgG_{2b} subclasses, respectively.

Table 15

Analysis of class and subclass of the antibodies of the present invention.

Antibody	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgA	IgM	κ
A1G5	—	+	—	—	—	—	+
E3H8	+	—	—	—	—	—	+
D2F4	—	—	+	—	—	—	+

[0249] v) Quantitation of OCIF by ELISA

[0250] Three kinds of monoclonal antibodies, ~~AIG5~~ A1G5, E3H8 and D2F4[[, which were]] obtained in EXAMPLE ~~25-iv,~~ 25-iv), were used as solid phase antibodies and enzyme-labeled antibodies, respectively. Sandwich ELISA was constructed by ~~each~~ different combinations of solid phase antibody and labeled antibody. The labeled antibody was prepared using an ~~Immuno Pure~~ IMMUNOPURE™ Maleimide-Activated Horseradish Peroxidase Kit (Pierce, Cat. No. 31494). Each monoclonal antibody was dissolved in 0.1 M $NaHCO_3$ at a concentration of 10 ~~μg~~ μg/ml, and 100 ~~μl~~ μL of the solution was added to each well of a ~~in~~ 96-well immunoplates (Nunc, MaxiSorp Cat. No. 442404) followed by allowing them to stand at room temperature overnight. Subsequently, each well ~~in~~ of the plate was blocked with 50% ~~Blockace~~ BLOCKACE™ (Snow Brand Milk Products, Co., Ltd.) at room temperature for 50 minutes, and ~~then was~~ washed three times with PBS containing 0.1% ~~Tween~~ TWEEN™ 20 (washing buffer).

[0251] A series of concentrations of OCIF was prepared by diluting OCIF with lst reaction buffer (0.2 M Tris-HCl buffer, pH 7.4, containing 40% ~~Blockace~~ BLOCKACE™ and 0.1% ~~Tween~~ TWEEN™ 20). Each well ~~in~~ of a 96-well immunoplate[[s]] was filled with 100 ~~μl~~ μL of the prepared OCIF solution with each concentration, allowed to stand at 37°C for 3 hours, and

subsequently washed three times with ~~the~~ washing buffer. ~~For dilution of~~ The POD-labeled antibody[[,]] was diluted 400-fold with 2nd reaction buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 25% ~~Blockace~~ BLOCKACE™ and 0.1% ~~Tween~~ TWEEN™ 20) ~~was used. POD-labeled antibody was diluted 400-fold with 2nd reaction buffer~~, and 100 ~~μl~~ μL of the diluted solution was added to each well of the immunoplates. Each immunoplate was allowed to stand at 37°C[[C]] for 2 hours, and subsequently washed three times with ~~the~~ washing buffer. After washing, 100 ~~μl~~ μL of a substrate solution (0.1 M citrate-phosphate buffer, pH ~~4. 5~~ 4.5, containing 0.4 mg/ml of o-phenylenediamine HCl and 0.006% H_2O_2) was added to each well ~~in~~ of the immunoplates and the immunoplates ~~were~~ incubated at 37°C for 15 min. The enzyme reaction was terminated by adding 50 ~~μl~~ μL of 6 N H_2SO_4 to each well. The optical density of each well was determined at 492 nm using an immunoreader (~~ImmunoReader~~ IMMUNOREADER™ NJ 2000, Nunc).

[0252] Using three ~~kinds of~~ different monoclonal antibod[[y]]ies ~~in~~ of the present invention, each combination of solid phase and POD-labeled antibodies leads to an accurate determination of OCIF concentration. Each monoclonal antibody ~~in~~ of the present invention was confirmed to recognize a different epitope of OCIF. A typical standard curve of OCIF using a combination of solid phase antibody, A1G5, and POD-labeled antibody, E3H8, ~~was~~ is shown in Fig. 14.

[0253] vi) Determination of OCIF in human serum

[0254] The concentration of OCIF in five samples of normal human serum was determined using an EIA system described in EXAMPLE 25-v). The immunoplates were coated with A1G5 as described in EXAMPLE 25-v), and 50 ~~μl~~ μL of the 1st[[.]] reaction buffer was added to each well ~~in~~ of the immunoplates. Subsequently, 50 ~~μl~~ μL of each human serum was added to each well ~~in~~ of the immunoplates. The immunoplates were incubated at 37°C for 3 hours and washed three times with washing buffer. After washing, each well ~~in~~ of the immunoplates was filled with 100 ~~μl~~ μL of POD-E3H8 antibody diluted 400-fold with the 2nd[[.]] reaction buffer and incubated at 37°C for 2 hours. After washing the immunoplates three times with ~~the~~ washing buffer, 100 ~~ul~~ μL of the substrate solution described in EXAMPLE 25-v) was added to each well and incubated at 37°C for 15 min. The enzyme reaction was terminated by adding 50 ~~μl~~ μL of 6 N H_2SO_4 to each well ~~in~~ of the immunoplates. The optical density of each well was determined at 492 nm using an immunoreader (~~ImmunoReader~~ IMMUNOREADER™ NJ 2000, Nunc).

[0255] 1st[[.]] reaction buffer containing the known amount of OCIF was treated in the same way and a standard curve of OCIF as shown in ~~fig.~~ Fig. 2 was obtained. Using the standard curve of OCIF, the amount of OCIF in human serum sample was determined. The results were shown in Table [[14]] 16.

Table [[14]] 16

The amount of OCIF in normal human serum

Serum Sample	OCIF Concentration (ng/ml)
1	5.0
2	2.0
3	1.0
4	3.0
5	1.5

EXAMPLE 26

[0256] Therapeutic effect on osteoporosis

[0257] (1) Method

[0258] Six week old [[M]]male Fischer rats[[, six weeks-old,]] were subjected to denervation of the left forelimb. These rats were assigned to four ~~groups(10 rats/group)~~ groups (10 rats/group) and treated as follows: group A, sham operated rats without administration; group B, ~~denervated~~ denerved rats with ~~intravenous administration of~~ the vehicle administered intravenously; group C, ~~denervated~~ denerved rats with OCIF administered intravenously at a dose of 5 ~~μ g~~ μg/kg twice a day; group D, ~~denervated~~ denerved rats with OCIF administered intravenously at a dose of 50 ~~μ g~~ μg/kg twice a day. After denervation, OCIF was administered daily for 14 days. After 2 weeks treatment, the animals were sacrificed and their forelimbs were dissected. Thereafter bones were tested for mechanical strength.

[0259] (2) Results

[0260] A [[D]]decrease in ~~of~~ bone strength was observed in control animals ~~of control groups~~ as compared to those animals of the normal groups while bone strength was ~~increase~~ increased in the group of animals that received 50 mg of OCIF per kg body weight.

[0261] Samples of the hybridomas that produce the claimed monoclonal antibodies were deposited in the National Institute of Bioscience and Human Technology National Institute of Advanced Industrial Science and Technology. The National Institute of Bioscience and Human Technology National Institute of Advanced Industrial Science and Technology accession numbers for the deposited hybridomas are:

Hybridoma Antibody Designation	Deposit Date	Accession No.
A1G5	2/5/01	FERM BP-7441
D2F4	2/5/01	FERM BP-7442
E3H8	2/5/01	FERM BP-7443

[0262] These deposits were made under the Budapest Treaty and will be maintained and made accessible to others in accordance with the provisions thereof.

[0263] The hybridomas will be maintained in a public depository for a term of at least 30 years and at least five years after the most recent request for the furnishing of a sample of the deposit is received by the depository. In any case, samples will be stored under agreements that would make them available beyond the enforceable life of any patent issuing from the above-referenced application.

[0264] Industrial availability

[0265] The present invention provides both a novel protein which inhibits the formation of osteoclasts and an efficient procedure for producing the protein. The protein of the present invention ~~has an activity to inhibit~~ inhibits the formation of osteoclasts. The protein will be useful for the treatment of many diseases ~~accompanying~~ accompanied by bone loss, such as osteoporosis, and as an antigen to ~~be used for~~ prepare antibodies useful for the immunological diagnosis of such diseases.

[0266] ~~Referring to the deposited the microorganism~~

~~Name and Address of the Depositary Authority~~

~~Name: National Institute of Bioscience and Human-Technology~~

~~Agency of Industrial Science and Technology~~

~~Ministry of International Trade and Industry~~

~~Address: 1-3, Higashi 1-chome, Tsukuba-shi, 1baraki-ken Ibaraki-ken, 305, JAPAN~~

~~Deposited date: June 21, 1995~~

~~(It was transferred from Bikkoken No. P-14998, which was deposited on June 21, 1995. Transferred date: October 25, 1995)~~

~~Accession Number: FERM BP-5267~~

A national deposit of the microorganism (accession number Bikkoken No. P-14998) was made on June 21, 1995, and transferred to the international depository named "National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology, Ministry of International Trade and Industry" having an address of 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305, JAPAN on October 25, 1995 as accession number FERM BP-5267, pursuant to the Budapest Treaty. The deposit is a Budapest Treaty deposit and will be maintained and made accessible to others in accordance with the provisions thereof. The international depository is currently known as "International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology".

[267] WHAT IS CLAIMED:

ABSTRACT

A protein which inhibits osteoclast differentiation and/or maturation and a method for ~~production of~~ producing the protein. The protein is produced by human embryonic lung fibroblasts and has a molecular weight of about 60 kD and about 120 kD under non-reducing conditions and about 60 kD under reducing conditions on SDS-polyacrylamide gel electrophoresis[[, respectively]]. The protein can be isolated and purified from the culture medium of ~~the said~~ fibroblasts. Furthermore, the protein can be produced by gene engineering. The present invention includes cDNA for producing the protein by gene engineering, antibodies having specific affinity for the protein or a method for ~~determination of~~ determining protein concentration using these antibodies.